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Role of cellulolytic enzymes in pathogenesis by Fusarium oxysporum f. lycopersici — Akhtar Husain and A. E. Dimond 329

Kansas aeromycology. VII. Smuts — S. M. Pady and C. L. Kramer 332

The severe bean mosaic virus, a new bean virus from Mexico — William D. Yerkes, Jr., and Graciano Patino 334

Predisposition of wheat seedlings to stem rust infection and development — Hosni A. Mohamed 339

Overwintering and migration of Ditylenchus dipsaci in organic soils of southern New York — G. D. Lewis and W. F. Mai 341

The effect of pyrimidines and 5-phenylazopyrimidines on the multiplication of tobacco mosaic virus — Toru Shimomura and Tokuzo Hirai 344

Purification by density-gradient centrifugation, electron microscopy, and properties of Cymbidium mosaic virus — M. K. Corbett 346

Streptomycin absorption, translocation, and retention in hops — Charles R. Maier 351

Control of vascular wilt diseases of carnation by culture-indexing — Paul E. Nelson, James Tammen, and Ralph Baker 356

Bacterial spot of tomato as influenced by temperature and by age and nutrition of the host — M. V. Nayudu and J. C. Walker 360

Genetics of resistance in Capsicum annuum to two virus diseases — A. A. Cook 364

Production of pectolytic enzymes and fusaric acid by Fusarium oxysporum f. conglutinans in relation to cabbage yellows — R. Heitefuss, M. A. Stahmann, and J. C. Walker 367

Oxidative enzymes in cabbage infected by Fusarium oxysporum f. conglutinans — R. Heitefuss, M. A. Stahmann, and J. C. Walker 370

Evolution of sexuality and pathogenicity. I. Interspecific crosses in the genus Helminthosporium — R. R. Nelson 375

Pathogenicity and stability of strains of Corynebacterium insidiosum — J. F. Fulkerson 377

Heat inactivation of stone fruit ringspot virus — George Nyland 380

Preparing nematode-free banana "seed" — Clive A. Loos and Sarah B. Loos 383

The influence of temperature on development of Phytophthora parasitica root rot of fuchsia — S. S. Patil and Roy A. Young 386

Radish yellows, a disease of radish, sugar beet, and other crops — James E. Duffus 389

Botryosphaeria ribis and its relation to a rot of apples — J. F. Fulkerson 394

Scopolin production in potato tubers infected with Phytophthora infestans — J. C. Hughes and T. Swain 398

PHYTOPATHOLOGICAL NOTE

Survival of stem rust urediospores on dry foliage of wheat — Hosni A. Mohamed 400

Book Listings 402

News

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ROLE OF CELLULOLYTIC ENZYMES IN PATHOGENESIS BY FUSARIUM OXYSPORUM F. LYCOPERSICI

Akhtar Husain and A. E. Dimond

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Accepted for publication November 23, 1959.

SUMMARY

Culture filtrates of *Fusarium oxysporum* f. *lycopersici* grown on various carbon sources were assayed for cellulase activity. Cellulase was detected when the fungus grew on cotton linters, wood cellulose, filter paper, or carboxymethyl cellulose as the sole source of carbon. Very little enzyme was produced on glucose-containing media. The fungus also produced the enzyme on living tomato stem sections suspended in water. A purified preparation of the *Fusarium* cellulase wilted young tomato cuttings. The enzyme is active at pH 2.0–9.0, with optimum around pH 6.0. It is inhibited by mercury but not by copper, zinc, or manganese ions. In pathogenesis by *Fusarium*, cellulase apparently releases nutrients for the fungus from host cell walls and also frees materials that induce wilting in the host.

Pathogenesis by *F. oxysporum* Schlecht. f. *lycopersici* Sacc. in the tomato involves more than one kind of action on the host. Part of the syndrome apparently arises from the action of vivotoxins. Another part evidently results from the hydrolytic action of fungal enzymes on host tissue (2, 3, 10, 12) and from polysaccharides arising from this process. *Fusarium* produces pectic enzymes in culture, and pectic enzymes of fungal origin have been detected in diseased but not in healthy plants (12). Culture filtrates from *Fusarium* and commercial preparations containing pectic enzymes produced wilting in tomato cuttings (3, 10) and also caused plugging of xylem vessels in the tomato stem (7). These filtrates and commercial preparations, however, were not pure. Hence, the symptoms produced cannot yet be ascribed to the pectic enzymes present.

In the pathogenesis of *Pseudomonas solanacearum* Erw. Smith, purified preparations of polygalacturonase do not wilt tomato cuttings, according to Husain and Kelman (4). A purified preparation of cellulase from *Myrothecium verrucaria* (Alb. and Schw.) Ditm. ex Fr. did cause wilting (4).

Several species of *Fusarium*, including some plant pathogens, have been reported to be cellulolytic (11). For this reason, the cellulolytic activity of the tomato wilt *Fusarium* and the role of cellulase in the disease syndrome have been investigated.

Materials and methods.—A highly pathogenic strain of *F. oxysporum* f. *lycopersici* was employed. The fungus was cultured on a modified Richard's solution containing 0.01% yeast extract. When glucose was the carbon source, it was added at a concentration of 1.5%. When a cellulosic material was the carbon source, it was added to the glucose-free medium at a concentration of 1%. Sources of cellulose used singly in the culture media were filter paper, wood cellulose (Solka Floc, Brown Co.), cotton linters (Hercules Powder Co.), carboxymethyl cellulose (CMC-30, Hercules Powder Co.), and chopped and washed tomato stems.

Two hundred ml of sterile medium in a 500-ml Erlenmeyer flask was inoculated and grown on a shaker for 4–6 days, after which mycelium was removed by filtration. The filtrate was centrifuged and the clear supernatant was used as the enzyme sample in studies on cellulase production.

Cellulase activity was determined by the loss in viscosity of solutions of carboxymethyl cellulose and also by the increase in reducing sugars on hydrolysis of a cellulose substrate. Viscosity was measured in a water bath at 32°C with a size-300 Fenske-Ostwald viscosimeter. The substrate was a 0.5% solution of carboxymethyl cellulose (CMC-70, with high viscosity and high substitution) containing citrate buffer at pH 5.5.

Reducing sugars were determined with Nelson's modification of the micromethod of Somogyi (6). One ml of culture filtrate was added to 9 ml of carboxymethyl cellulose solution (CMC-30) in 0.2*M* acetate buffer so that final pH was 5.5 Final concentration of the substrate in the reaction mixture was 0.5%. One-ml aliquots were removed at the beginning and at the end of 2 hours, and the glucose present was determined. Enzyme activity was expressed as the number of mg glucose appearing per ml of reaction mixture during this period.

To investigate the effect of cellulase on tomato cuttings, the cellulase was purified by a method similar to that of Whitaker (13). Five liters of culture filtrate were concentrated to 500 ml by distillation at 30°C under reduced pressure. To the concentrated filtrate was added 225 ml of saturated ammonium sulfate solution and 25 ml of 1*M* phosphate buffer. After standing overnight, the solution was centrifuged and the sediment discarded. The supernatant was placed in a large beaker in which was suspended a cellophane bag packed with finely powdered ammonium sulfate. After 4 days at 5°C the solution was saturated with ammonium sulfate and centrifuged, and the supernatant discarded. The sediment was dissolved in 100 ml of distilled water, at which point it contained 75% of the original enzyme activity. To this extract was added 4 volumes of saturated ammonium sulfate. The solution was centrifuged and the supernatant discarded. The sediment was dissolved in 100 ml distilled water and was dialyzed against distilled water for 48 hours to remove the ammonium sulfate. The dialyzed sample was stored at 5°C until wilt tests were made.

Results.—*Cellulase activity in relation to pH of the substrate.*—The rate of hydrolysis by the enzyme was determined in the range pH 2.0–9.0. Citrate buffer

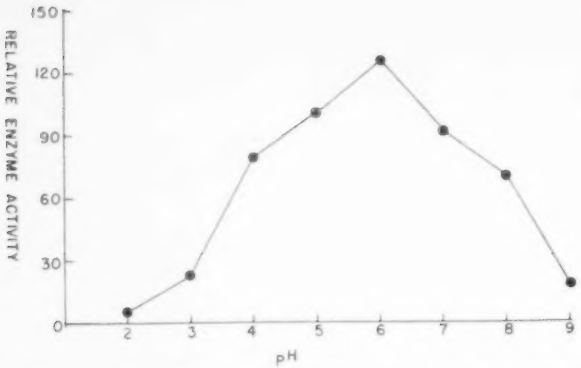

Fig. 1.—The effect of pH on actvity of the cellulase of *Fusarium oxysporum*, f. *lycopersici*.

was used below pH 7.0, and borate buffer above. Relative activity was expressed as the reciprocal of the time in minutes for 50% loss in viscosity of the substrate. The enzyme was active over this entire range, with optimum activity around pH 6.0 (Fig. 1).

Cellulase production.—The culture filtrate obtained from media containing glucose showed only weak cellulase activity. The fungus produced a large amount of enzyme on all media containing cellulose. Thus, a 1:4 dilution of a filtrate from a culture grown on carboxymethyl cellulose caused rapid loss in viscosity of the substrate (Fig. 2). This preparation also produced 0.2 mg of glucose per ml of reaction mixture whereas a 0.25% solution of a commercial cellulase (Enzyme AP 19, supplied by Rohm & Haas Co., Bristol, Pa.) produced 0.56 ml of glucose per ml within the same period.

Cellulase activity was highest when *Fusarium* grew on chopped tomato stem as a carbon source. Table 1 compares this cellulase activity with that on other media. Although enzyme yields were greatest on cellulose-containing media, mycelium was produced most abundantly on media containing glucose.

The ability of the fungus to degrade native cellulose was studied by inoculating filter paper strips suspended in a nutrient solution free of any other carbon source. The strips were completely disintegrated within a week.

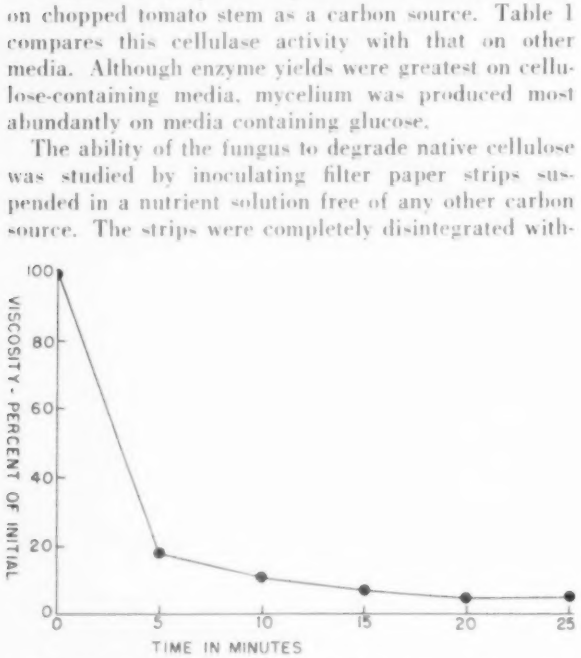

Fig. 2.—Reduction in viscosity of carboxymethyl cellulose solution caused by a diluted culture filtrate (1:4) of *Fusarium oxysporum* f. *lycopersici*.

The cellulase is also produced on sections of host stem tissue. Half-inch-long sections of 6–8-week-old tomato plants were sterilized with propylene oxide vapor for 12 hours. These sections were placed in sterile flasks each containing 50 ml of sterile distilled water. The flasks were seeded with a spore suspension of *Fusarium*. After 3–4 days of growth, the stem pieces were squeezed through cheese cloth, the extract was centrifuged, and the supernatant was assayed for enzyme activity. A similar extract was made from uninoculated stem sections. Weak cellulase activity was detected in inoculated stem sections, but no enzyme was present in uninoculated tissue (Fig 3). When these sections were placed on nutrient media after the incubation period and before extraction, the inoculated sections yielded only *Fusarium* and the uninoculated ones were sterile.

Effect of purified cellulase from Fusarium on tomato cuttings.—The purified dialyzed enzyme solution was diluted with distilled water so that enzyme activity was the same as in the crude culture filtrate. Ten ml of this diluted solution was placed in 15-ml vials. Stems of 6-in. seedlings of Bonny Best tomato were cut under water and inserted into the vials. Heat-inactivated enzyme solution and sterile distilled water were used as controls. The seedlings were incubated at 25°C. Five seedlings were used for each treatment, and the experiment was repeated 3 times with the same results. The leaflets of the cuttings in active enzyme lost turgor in 16 hours. These cuttings were completely wilted at 20 hours. Petioles were less severely affected than leaves. The wilted cuttings were removed from the enzyme solution and the bases of the stems were trimmed and placed in water. These plants showed only partial recovery of petioles in 24 hours. No injury was noticed in the cuttings placed in heat-inactivated enzyme or water, even at 48 hours.

Inhibition of the enzyme.—The effect of inhibitors on *Fusarium* cellulase was studied by mixing the test compound with the substrate, adding the enzyme, and measuring the rate of change in viscosity of the substrate. Of the various metallic ions used, mercuric ion inhibited cellulase activity at a concentration of $10^{-3}M$, but copper, zinc, and manganese ion had no effect. Although reported to be inhibitors of other fungal cellulases, zinc ethylenebis[dithiocarbamate] (1, 9) and 8-quinolinol sulfate (5) did not inhibit the *Fusarium* cellulase.

Table 1.—Effect of carbon sources on cellulase production by *F. oxysporum* f. *lycopersici*

Carbon source	Relative enzyme activity[a]
Glucose	7
Cotton linters	15
Filter paper	140
Wood cellulose	1110
Carboxymethyl cellulose (CMC-30)	1425
Chopped tomato stem	2500

[a] Activity expressed as the reciprocal of the time (minutes) × 1000 for 50% loss in viscosity of substrate.

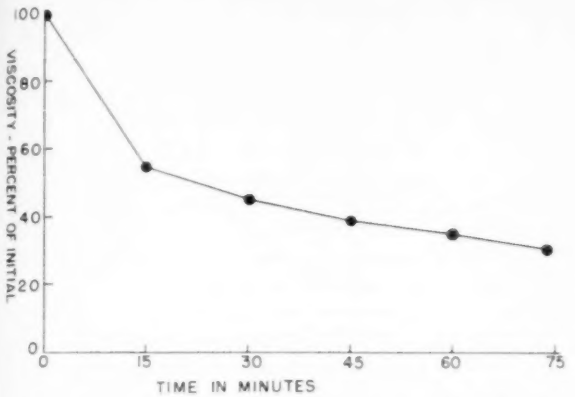

Fig. 3.—Reduction in viscosity of carboxymethyl cellulose solution caused by extract from inoculated tomato stem sections.

Discussion.—The present investigation demonstrates that *F. oxysporum* f. *lycopersici* is a cellulolytic fungus and is capable of degrading native cellulose (filter paper, cotton linters, and wood cellulose) and derived soluble cellulose (carboxymethyl cellulose). Thus, according to the hypothesis of Reese (8), *Fusarium* produces both the C_x and the C_1 enzymes. That *Fusarium* produces these enzymes on living tomato tissue suggests that it may also produce cellulase in infected tomato plants.

The present investigation has demonstrated the ability of cellulase preparations, acting on substrates in the tomato stem, to cause wilting in tomato cuttings. As yet, cellulase has not been detected in the vascular system of *Fusarium*-infected tomato plants, but its presence is highly probable. Cellulase presumably induces wilting in tomato cuttings in a manner similar to that proposed for pectic enzymes of the polygalacturonase type. Thus cellulase, liberated by *Fusarium* into the transpiration stream, will encounter its normal substrate on vessel walls and hydrolyze the cellulose partially to large molecules of a hemicellulosic nature. These, when freed into the transpiration stream in moderate quantity, interfere with normal water movement to leaves and cause wilting.

The role of the pectic enzymes in the pathogenesis of *Fusarium* wilt needs further examination in light of the present work. The crude enzyme samples and the alcoholic precipitate used by Gothoskar et al (3) may well have contained some cellulase. The commercial pectic enzyme used by these workers (Pectinol 100D) is known to contain some cellulase activity. Both the pectolytic and the cellulolytic enzymes are presumably involved in pathogenesis, but the contribution of each type can be determined only with purified preparations to which plant tissue is exposed under rather specific conditions. The *Fusarium* cellulase used in the present investigation was at least partially purified and was completely free of any pectic enzyme, as indicated by its failure to attack pectin.

In light of the present study, the action of cellulase produced by *Fusarium* may be conceived to act in pathogenesis in three ways. First, it is apparently involved in wilt induction. Second, the hydrolytic products of cellulase activity may provide *Fusarium* with carbohydrate for its continued development in the host, as the action of the pectolytic enzymes may also do. Third, the cellulase of *Fusarium* is probably also involved in the escape of the pathogen from vascular tissue in advanced stages of disease when the host is in dying condition. Under these conditions, the host tissue will probably be depleted of sugars, cellulase and pectolytic enzymes will be produced in high quantity, and as a result the fungus will rapidly disintegrate the dead host tissues.—The Connecticut Agricultural Experiment Station, New Haven.

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KANSAS AEROMYCOLOGY. VII. SMUTS

S. M. Pady and C. L. Kramer

Respectively: head, botany department, and assistant professor of botany. The assistance of graduate students A. R. Bhatti and V. K. Pathak, and the technical assistance and cooperation of Laurel Ouye, Joyce Murphey, Dorothy Bell, and Jeanette Arnett are gratefully acknowledged.

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SUMMARY

Two groups of smut chlamydospores were common in Kansas air in 403 daily samples between September, 1956, and August, 1958. One group of small (5–7 μ) smooth spores was from either *Ustilago hordei* (covered smut of oats, barley, rye) or *Sphacelotheca sorghi* (kernel smut of sorghum); the other was from *U. maydis* (corn smut). Numbers, though seasonal, with peaks in June and July, were substantial on some winter days. Numbers were 5–371/cu ft (usually fewer than 100/cu ft) in summer, and 1–23/cu ft in winter. Smut colonies were present on exposed agar plates but could not be correlated with chlamydospore concentration.

Introduction.—The presence of spores of saprophytic and parasitic fungi in the air is well established. Smut chlamydospores, a common constituent of this airborne fungal flora, have been reported over agricultural lands in Canada (5), England (2), the United States (9), and the Arctic (14), and over the Arctic Ocean (11), Pacific Ocean (7), Atlantic Ocean (12), and Mediterranean Sea (18). Most of the early work was qualitative. Recent work in England and Canada has been quantitative, but few quantitative data are available on smut spore concentrations in the United States. Pady (9) found smut spores to be seasonal in preliminary quantitative studies in Kansas in 1953–55. From September, 1956, to August, 1958, a detailed study was made of the fungus flora of the air, using cultures in addition to spore-trapping techniques (16). Materials, methods, and general results have been reported (6).

Results and discussion.—Slides and plates were exposed daily in a slit sampler (8) at 8:30–9:30 A.M. on the roof of a campus building. The most common spores in the air were conidia of *Cladosporium* spp. (40.9%), basidiospores (24.3%), yeast cells (7.3%), and smut chlamydospores (5.9%). This paper presents data on the seasonal distribution and spore concentration of the smuts.

The chlamydospores fell in 2 groups: 1) 5–8 μ, round, thick-walled, smooth, and light brown; and 2) 7–10 μ, round, thick-walled, echinulate, and darker brown. The first group was assigned to *Ustilago hordei* (Pers.) Lagerh. (covered smut of barley, oats, rye) and *Sphacelotheca sorghi* (Lk.) Clint. (kernel smut of sorghum), which are indistinguishable by spore morphology. The second group was identified as *U. maydis* (DC.) Cda. (corn smut). No records were kept of the numbers of these two groups, but the former were much more numerous. Chlamydospores of *Tilletia* and other unidentified smuts were observed on one or two occasions. Groups or clusters of spores of 2–20 were common; in the early work no records were kept of the number of groups.

Smut chlamydospores were present throughout the year (Fig. 1) with maximum numbers in June and July and lowest numbers from January to April. In the winter of 1957, spores were present on all but 6 days, whereas in the following winter they were present only on 6 days.

There was great day-to-day variation throughout the year. June 16–20, 1957, numbers were 12.4, 40.3, 299.5, 9.3, and 94.5/cu ft. Heavy rains June 18 removed most of the spores from the air. The highest concentration of spores was 371.2/cu ft obtained July 5, 1958; yet on July 4 the number was only 6.2/cu ft.

Smut-like colonies were fairly common on the plates, totalling 680 colonies. On the Rose Bengal-Streptomycin medium, used routinely (16), characteristic colonies were produced: small, raised, ridged to convolute, white, with yeastlike consistency, and made up of budding yeastlike cells. Comparison with known cultures of species of *Ustilago* and *Sphacelotheca* confirmed their identification as smuts, but generic and specific identifications were not possible. The highest number of colonies was in October, 1957 (74), with high numbers also in December (56) and March (55). Colonies were present throughout the year but their distribution was not correlated with chlamydospore concentration. The highest number of colonies was in the period Aug.–Dec. 1957, with monthly totals of 18, 35, 74, 19, and 56. Chlamydospore numbers for these months were 13.5, 6.5, 7.1, 1.0, and 4.4/cu ft. In January, 1958, no smut spores were observed on the slides, yet 31 colonies were present on the plates. The highest number of chlamydospores was in June, 1958 (Fig. 1), and it would be expected that colony numbers would be correspondingly high, but only 5 colonies were found. No explanation for these inconsistent results is known. The medium used is satisfactory for most fungi (16), but is apparently unfavorable for cereal smut chlamydospores.

Cereal smuts are abundant in Kansas (10) and the presence of high numbers of chlamydospores coincides with the blossoming and harvesting of cereals, when most chlamydospores would be expected to be set free. There is a gradual decline in the fall and a gradual increase in the spring. The occasional days in spring, fall, and winter when numbers are high (Fig. 1) are correlated with high winds.

Smut chlamydospores are not limited to agricultural areas; they have been found at altitudes of 9000 feet over Canada and the Yukon in numbers to 17.3/cu ft (11). Smut spores were abundant (0.1–19.5/cu ft) at

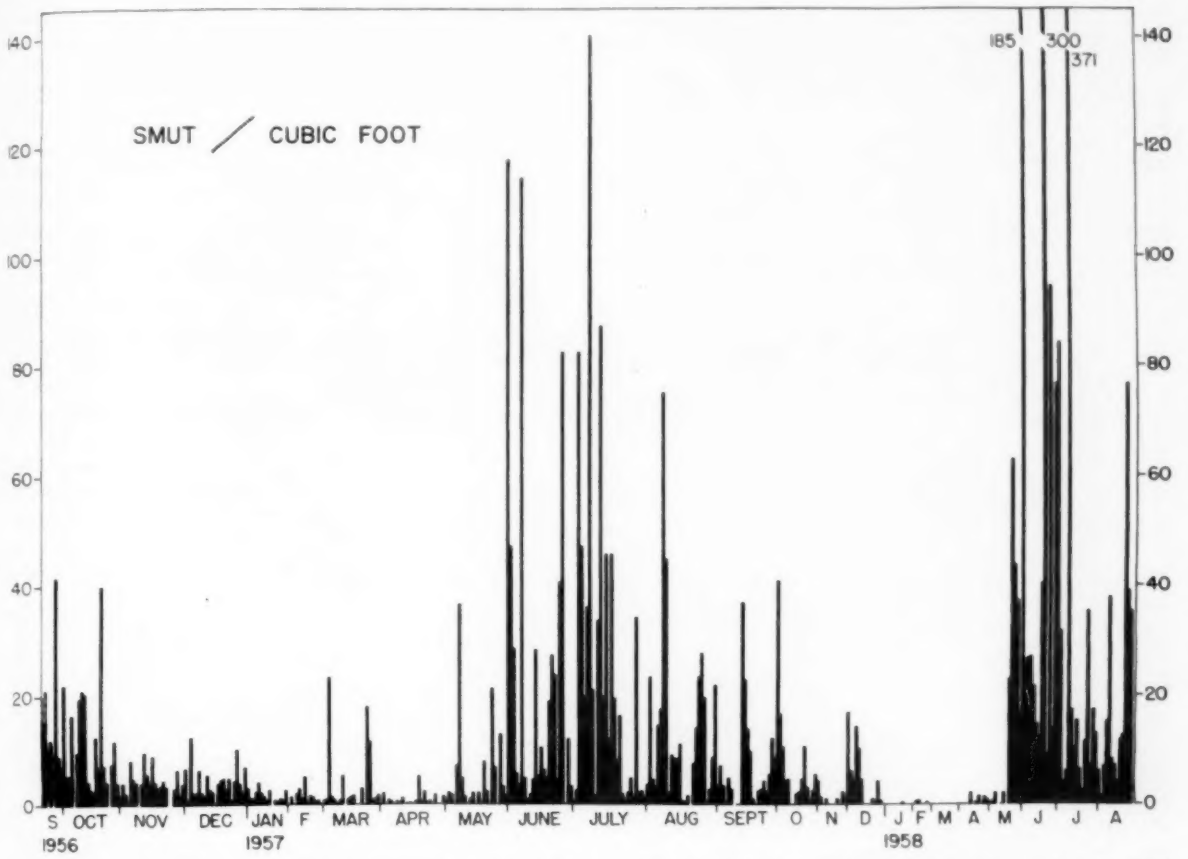

Fig. 1.—Number of smut chlamydospores per cu ft of air collected on each sampling day from September 16, 1956, to August 31, 1958. Clusters of spores are not recorded.

Churchill, Manitoba, in a series of ground-level samples taken July 23 to August 12, 1950 (14). In a flight to Resolute Bay, 1000 miles north of Churchill, on August 1, 1950, smut spores were present in all samples in concentrations of 0.12–8.2/cu ft (14). In a flight over the North Pole under winter conditions, in March, 1949, smut spores were collected: 6 smut chlamydospores among 14 spores obtained in 15-hour-long exposures (11). Smut spores were reported from flights in June and August over the Atlantic Ocean (12); they were abundant in tropical air masses (to 4.5/cu ft), and less so in polar air. Their presence over the Mediterranean in exposures made from the deck of a ship was recently reported (18).

Recent studies with quantitative samplers give data on spore concentrations. In England, Hirst (4) found numbers varied from 100/m^3 to 6000/m^3 from July 21 to 25, 1951. Gregory and Hirst (2) observed that *Ustilago* chlamydospores reached a peak in June and July, with numbers to 1500/m^3. Pady and Kapica (13), working on the roof of a city building in Montreal from October, 1954, to December, 1955, found smut spores in every month except December, with peaks to 112/cu ft in July and August.

Diurnal periodicity of *Ustilago* spores in England with a peak in the afternoon (4) has been confirmed (1, 2, 3, 17). Panzer et al (15) found a similar periodicity in the United States, with spores of an unidentified smut genus more abundant in the day than at night. Current (unpublished) studies in Kansas generally confirm the above observations: on most days, smut spores had an afternoon peak; on some, a morning peak; and occasionally, a night peak. During an all-day series on November 17, 1958, an unusually high reading of 602 spores/cu ft was obtained at 7:30 A.M. Since the data herein are based on a single daily mid-morning sample, the numbers given in Fig. 1 may not present a true picture of the spore load. They indicate, however, that chlamydospores of *Ustilago* and *Sphacelotheca* are present throughout the year, with peak numbers in June and July.—Department of Botany and Plant Pathology, Kansas State University, Manhattan.

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THE SEVERE BEAN MOSAIC VIRUS, A NEW BEAN VIRUS FROM MEXICO

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SUMMARY

A new mosaic disease of beans from Mexico has field symptoms similar to those of Common Bean Mosaic Virus, but more severe. Mechanically inoculated leaves develop local lesions as necrotic spots, vein necrosis, or ring spots, dependent on the variety. All inoculated plants develop systemic leaf mottle. The host range includes all tested varieties of *Phaseolus vulgaris*, *P. lunatus*, *Glycine max*, and *Vigna sinensis*. Symptomless carriers include *Lathyrus odoratus*, *Pisum sativum*, and *Vicia sativa*. *Cucumis* sp., *Lycopersicum esculentum*, and *Nicotiana* spp. are insusceptible. The virus is not seed-borne. It withstands heating to 92°C, dilution to 1:4,000,000, and aging of over 7 months in dried tissues, 11 weeks as expressed juice, and 10 weeks as frozen juice. The virus is also quite resistant to treatment with protein-precipitating and oxidizing agents.

There have been only a few references to bean viruses in Mexico. These have been general and without specific reference to the type of symptoms, strains of the virus, or the bean races or varieties attacked (2, 3, 4, 5). For the last 4 years a mosaic type of virus disease of beans has been observed in the Huasteca (Gulf Coast) region of Mexico. The disease appears widely distributed, causing small losses each year. Studies were undertaken in 1956 to determine the virus inciting the disease and appropriate control measures. The results are presented herein.

Symptoms.—Symptoms in the field appear very similar to those incited by the common bean mosaic virus (CBMV) or its variant (New York #15). Included are leaf mottle typical of mosaics (Fig. 1-A), leaf and pod distortion as blistering and puckering of the leaves and twisting of leaves and pods, epinasty (on certain varieties), dwarfing and rosetting as shortening of internodes, and poor seed set. Artificially inoculated leaves of some varieties also exhibited local necrotic lesions as small (1 mm) roughly circular spots on the majority of bean varieties tested, veinal necrosis on the variety Scout (Pinto 1534), and ring spots on Dark Red Kidney, Michelite, Mosaic Resistant Navy 3593, Sanilac, and Wade varieties (Fig. 2). In all cases, bean plants exhibiting local lesions later developed leaf mottle throughout the plant, typical of systemic mosaic infection. Inoculum taken from systemically mottled leaves with no local lesions produced local lesions on locally inoculated primary leaves on plants of the same variety. Inoculations with juice extracted from systemic mottle leaves and treated at temperatures in excess of 80°C, or diluted to 1:4,000,000, continued to produce both local lesion and systemic mottle symptoms. Although we cannot definitely rule out the possibility of a mixture of two viruses, the above two facts greatly reduce this possibility.

Experimental results.—A number of infected plants were taken to the Chapingo (Mexico) laboratory of the Oficina de Estudios Especiales, S.A.G. Mechanical inoculation with carborundum as an abrasive was successful. Local lesions appeared within 4 days, and systemic infection within 15 days. Some Mexican lines became necrotic and died about 15 days after inoculation (Fig. 1-C).

The reactions of certain bean varieties resistant to CBMV and its variant and the severity of symptoms caused by the Mexican virus (Fig. 1-B), led to studies to determine host range, physical properties, the effects on virus activity of alcohol, bichloride of mercury, and hydrogen peroxide, and cross protection with CBMV and its variant.

Host range.—A number of varieties of *Phaseolus vulgaris* and other legumes and species in other families were inoculated with the Mexican virus. The

results of these inoculations, enumerating hosts, symptomless carriers, and insusceptible species, are presented in the section headed "Hosts" at the end of the paper.

Inoculations made with juice from various vegetative plant parts showed that the virus was present throughout infected plants, including the roots.

Transmission. — Preliminary studies indicate that

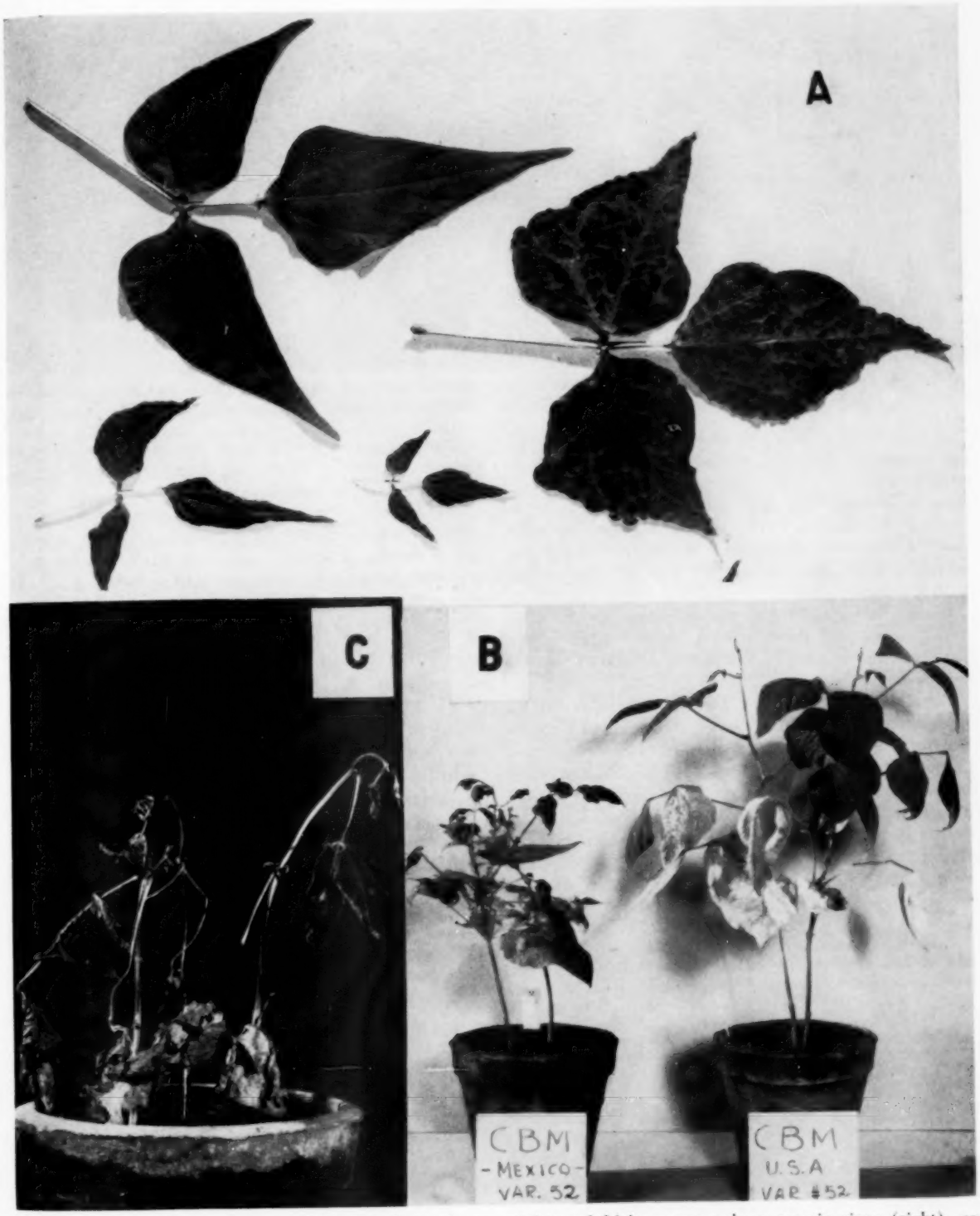

Fig. 1.—A) Foliage symptoms of Mexican bean virus. Normal leaf at upper left. All leaves about same age, from different plants. **B)** Comparison of Mexican bean virus (left) and Idaho common bean mosaic virus (right), on variety Mexico #52 16 days after inoculation. **C)** Lethal necrosis of Mexican bean virus on variety Cacahuate #1.

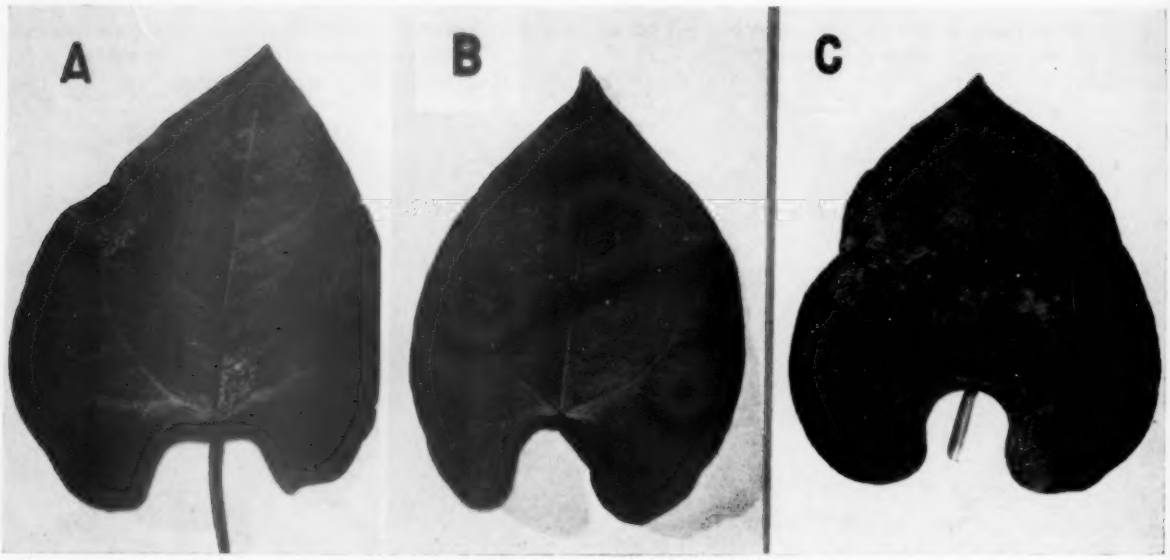

Fig. 2.—Primary symptoms on mechanically inoculated leaves: **A)** vein necrosis on variety Scout (Pinto 1534), **B)** necrotic spots on variety #52 (Mexican), **C)** ring spots on variety Michelite.

seed transmission does not occur in any host studied. The nature of the field spread implicates insects as possible vectors, but leafhoppers (*Empoasca kraemeri* Ross & Moore), the most abundant insect pest of beans in the Huasteca area, did not transmit the virus under greenhouse conditions. Studies are being conducted to determine the possible role of other insects in this regard.

Besides inoculation with carborundum, grafts of infected stems and petioles onto healthy bean plants transmitted the virus. Symptoms appeared on the root-stock plants within 2 weeks.

Physical properties.—1) *Dilution endpoint.*—Juice expressed from trifoliate leaves showing good mottle symptoms was diluted with distilled water to various strengths. No buffer was used. The diluted virus extract was inoculated to the Mexican bean variety Blanco 157, a good local-lesion indicator. Local lesions and subsequent systemic mottle were produced at dilutions up to 1:4,000,000. No higher dilutions were made. Table 1 shows the results with some of the dilutions. Not all leaves inoculated developed symptoms. The results show the average number of lesions obtained. Plants not developing local lesions did not develop mottle symptoms.

Table 1. Local-lesion counts of the Mexican bean virus after various dilutions, on variety Blanco 157.

Dilution (virus/water)	No. leaves inoculated	Av. no. local lesions per inoculated leaf*
1:100	32	<1000
1:50,000	30	80
1:100,000	32	25
1:500,000	24	25
1:1,000,000	30	6
1:2,000,000	30	4
1:3,000,000	32	5
1:4,000,000	40	5

* Not all leaves inoculated developed symptoms. Results show number of local lesions/number of leaves inoculated.

2) *Thermal inactivation.*—Five-ml samples of fresh plant juice extracted from trifoliate leaves showing good mottle symptoms were subjected to heat in a clinical constant-temperature water bath. After 10 minutes at each temperature range, the juice was inoculated to *P. vulgaris*. The results are in Table 2. Infectivity was retained at temperatures up to 92°C. Again, all plants demonstrating local lesions subsequently developed systemic mottle.

3) *Effects of aging.*—Three tests were made of aging of extracted virus. Extracted juice diluted 1:10 was frozen and stored at −7°C. At regular intervals, samples were removed, thawed, and inoculated to *P. vulgaris*. The virus extract maintained its virulence during 10 weeks of such storage, and infected all plants inoculated.

Another set of virus extracts was kept at room temperature in well-stoppered test tubes without added preservative. After 11 weeks such juice infected all plants inoculated.

A number of infected trifoliate leaves were dried and stored at room temperature. At monthly intervals 1 g of the dried material was ground with 10 ml of

Table 2.—Effects of heat treatment on activity of the Mexican bean virus.

Treatment °C (±0.5°)	% infected plants
45	100
56	100
65	83
75	100
85	93
90	63
92	56
92.5–93	0

Table 3.—Effects of various chemicals on the Mexican virus, mixed in varying proportions.

Proportion (Virus/chemical)	% infected plants		
	$HgCl_2$ (1:1000)	Ethanol (96%)	H_2O_2 (3%)
1:0	100	100	100
20:1		100	
10:1		100	100
5:1		100	
1:1	100	100	100
1:2		83*	100*
1:4		60*	100*
1:6		60*	
1:10	100*	50*	100*
1:20	25*	50*	100*

* Precipitate formed on mixing.

distilled water and inoculated to plants of *P. vulgaris*. The dried material maintained virulence up to 7 months, the longest period tested.

Effects of chemicals.—Samples of extracted juice from trifoliate leaves with good mottle symptoms were mixed with bichloride of mercury (1:1000), ethanol (96%), and hydrogen peroxide (3%) in various proportions. The juice was added to the chemical, well mixed, and after 10 minutes was inoculated to the variety Blanco 157. The results are shown in Table 3. As can be seen, the virus is quite resistant to protein precipitation (ethanol and bichloride of mercury) and to oxidation (hydrogen peroxide).

Cross-protection test.—Tests were conducted to determine if the Mexican virus showed a cross-protection relationship to CBMV and its variant. The test varieties used were 3 Mexican bean varieties known to show local-lesion (necrotic spot) reaction to the Mexican virus and known to be susceptible to CBMV and its variant. These were inoculated with the latter viruses (Idaho collections). When the trifoliate leaves became mottled, they were inoculated with the Mexican virus. In all cases, including plants not inoculated with CBMV or its variant, local lesions appeared on inoculated leaves. No cross-protection relationship was shown.

Discussion.—These results lead us to believe that we are dealing with a previously undescribed bean virus. The symptoms, although similar to those of other bean mosaic diseases, are more severe. The constant association of local lesions at the site of mechanical inoculation and subsequent development of systemic symptoms is, we believe, unique with bean mosaic viruses. The host range includes all tested varieties and lines of *P. vulgaris*, including varieties reported resistant to other bean mosaic viruses, e.g., Great Northern 123, Great Northern 31, Columbia Pinto, Ranger, Rival, etc. (7, 8, 9, 10) as well as a number of other legume species. The dilution endpoint of 1:4,000,000 is far in excess of that reported for any other bean mosaic virus. The thermal inactivation temperature also is higher than for any other bean virus, except southern bean mosaic virus (SBMV). The long maintenance of virulence *in vitro*, especially as dried material, is another property known for few bean viruses. Cross-protection tests indicate no close relationship to CBMV or its variant. The resistance to inactivation by heat, aging, dilution, and chemical treatment indicates its stability. The Mexican virus is compared with other known bean mosaic viruses in Table 4.

Though the Mexican virus is similar to other bean mosaic viruses, it differs in one or more characteristics. In some properties it appears similar to SBMV or a strain of tobacco mosaic virus (TMV). The susceptibility of such hosts as *Melilotus indica*, *Phaseolus multiflorus*, and *Vigna sinensis* and the high dilution endpoint seem to eliminate SBMV. The lack of symptoms on such hosts as *Capsicum annuum*, *Cucumis* sp., *Lycopersicum esculentum*, and *Nicotiana* spp. appears to eliminate the possibility that it is a strain of TMV. A combination of symptoms produced, host range, and

Table 4.—A comparison of various bean mosaic viruses with the Mexican bean virus.[a]

	Dilution end-point	Thermal inactivation temperature	Aging limit *in vitro*	Transmissions[b]	Symptoms on bean[c]
Common mosaic	1:1000	56–58°C	32 hours	MSI	SM,LL,BR
Common mosaic variant	1:1000	56–58°C	32 hours	MSI	SM,LL
Yellow mosaic	1:2000	58–60°C	32 hours	MI	SM,LL,N
Alfalfa mosaic	1:3000	65–70°C	4-5 days	MI	SM,LL
Alfalfa yellow mosaic	1:5000	65–70°C		MI	SM,LL,N
Pod mottle	1:100,000	70–75°C	93 days	MI	SM,LL
Southern mosaic	1:500,000	90–95°C	32 weeks	MS[d]	SM or LL
Yellow stipple	1: 75,000	72–75°C	5 days	M	SM
Pea mosaic #4	1: 8,000	62–65°C	2 days	M	SM
Pea mosaic #5	1: 8,000	62–65°C	2 days	M	SM
Pea virus #2	1:100,000	62–64°C	5 days	M	SM
Mexican virus	1:4,000,000	90–92.5°C		MS	SM,LL,N
dried			7 mo.		
juice			11 wk.		
frozen			10 wk.		

[a] Data from Zaumeyer (6,9).
[b] M, mechanical; S, seed; I, insect
[c] SM, systemic mottle; LL, local lesions; BR, black root; N, necrosis.
[d] Seed transmission is limited.

physical properties appears to eliminate tobacco necrosis virus (bean stipple streak). We therefore propose that it be recognized as a new virus of beans, to be called Severe Bean Mosaic Virus (SvBMV). We also propose the following epithet, according to the binomial system of virus nomenclature (1):

***Marmor mexicanum* n. sp.**

Common name: Severe Bean Mosaic Virus (SvBMV)

Hosts: *Phaseolus vulgaris* L. Varieties tested and found susceptible: Black Valentine, Blue Lake, Bountiful, California Pink, California Pinto, California Red, Columbia Pinto, Contender, Dark Red Kidney, Florida Belle, Great Northern U.I. 31, Great Northern U.I. 123, Great Northern 1140, Great Northern Montana 5, Idaho Bountiful, Light Red Kidney, Logan, Michelite, Michigan Cranberry, Mosaic Resistant Navy 3593, Potomac, Pinto U.I. 111, Pinto U.I. 78, Pinto 1540, Pinto 2207, Ranger, Red Mexican 34, Red Mexican 35, Red Mexican U.I. 3, Resistant Asgrow Valentine, Refugee U.S. 5, Rival, Sanilac, Scout (Pinto 1534), Small White, Stringless Green Refugee, Tendergreen, Tenderlong 15, Topcrop, Wade, White Kidney, Yellow Eye, and Mexican varieties Canario 101, Canocel, and line #52.

Other susceptible legumes are: *Cyamopsis* sp., *Glycine max* Merr. (varieties Ogden, S-100), *Melilotus indica* All., *Phaseolus acutifolius* A. Gray, *P. lunatus* L. (varieties Early Thorogreen, Fordhook 242, Ventura, and 4 wild Mexican collections), *P. multiflorus* Lam., *P. coccineus* × *P. vulgaris* hybrid (F_2), *Trifolium alexandrinum* L., and *Vigna sinensis* Endl.

The following species are symptomless carriers of the virus, i.e., virus was recovered from the upper leaves subsequent to inoculation on primary leaves but no effects of the virus were noted: *Canavalia* sp., *Cologania* sp., *Lathyrus odoratus* L. (variety Cuthbertson), *Phaseolus calcaratus* Roxb., *P. coccineus* L., *P. lathyroides* L., *P. mungo* L., *Pisum sativum* L. (varieties Alaska, Alderman, Little Marvel, Perfection, and World's Record), *Teramnus* sp., and *Vicia sativa* L.

Insusceptible species tested: *Cajanus indicus* Spreng., *Capsicum annuum* L., *Centrosema pubescens* Benth., *Chenopodium* sp., *Crotalaria anagyroides* H.B.K., *C. spectabilis* Roth, *Cucumis* sp., *Desmodium* sp., *Indigofera* sp., *Lathyrus cicera* L., *L. clitorus* L., *L. strictus* Graver, *Lycopersicum esculentum* Mill., *Medicago sativa* L., *Melilotus alba* Desr., *Nicotiana glauca* Graham, *N. glutinosa* L., *N. rustica* L., *N. tabacum* L., *Phaseolus adenatus* G.F.W. Mey., *Sesbania* sp., *Solanum tuberosum* L., *Stizolobium deeringianum* Bort., *Trifolium pratense* L., and *Vicia villosa* Roth.

Locality: State of Veracruz, Mexico. Type locality, Cotaxtla, Veracruz.

Symptoms: Leaf distortion, epinasty of primary and trifoliate leaves, chlorotic mottling, dwarfing, pod distortion, abortive seed set, local lesions as necrotic spots, vein necrosis, or ring spots on inoculated leaves of *Phaseolus vulgaris*. Some varieties react with complete necrosis and death.

Transmission: By mechanical inoculation of expressed juice and by grafting. Not seed-transmitted by species tested. Possibly insect-transmitted. Natural means of transmission not known.

Immunological relationships: No cross-protection with common bean mosaic virus (CBMV) or its variant (New York #15).

Thermal inactivation: 10 minutes at 92–92.5°C.

Dilution: Withstands dilutions up to 1:4,000,000.

Aging: Survives more than 7 months in dried tissue, 11 weeks as expressed juice, and 10 weeks as frozen juice.

Chemical treatment: Withstands mixing with ethanol (96%) up to 1 part extract to 20 parts ethanol; 1 part extract to 20 parts $HgCl_2$ (1:1,000); and 1 part extract to 20 parts H_2O_2 (3%).

Virus name taken from Mexico, country of type locality; genus pertains to Holmes' group "A" of genus *Marmor*, those mosaic viruses relatively resistant to heat inactivation (1).—Rockefeller Foundation Agricultural Program, Calle Londres 40, Mexico 6, D.F., Mexico.

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PREDISPOSITION OF WHEAT SEEDLINGS TO STEM RUST INFECTION AND DEVELOPMENT

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SUMMARY

Pre-inoculation temperatures affected the number of infection centers and stem rust reaction. More infection centers were produced and infection types were higher if the pre-inoculation temperature was 85°F than if it was 70°F.

Several workers report that different post-inoculation temperatures give different rust reactions (2, 3, 4, 5). High temperature after inoculation often increases uredium size and changes temperature-sensitive wheat varieties from resistant to susceptible.

Little has been done to study the effect of temperature prior to inoculation on the severity of diseases. Dickson (1) found that wheat seedlings were susceptible to *Gibberella saubinetii* (Mont.) Sacc. if soil temperature was high (12–28°C) in the early stages of host development, but no seedling blight occurred if soil temperature was below 12°C. Corn was easily infected by the same fungus if soil temperature was low (8–20°C), but no blight occurred above 24°C. Wheat developed better at the low temperature, and corn at the high temperature. In 1944, Straib and Noll (7), working with *Puccinia rubigo-vera* (DC) Wint. f. sp. *tritici* (Eriks.) Carleton and *P. glumarum* (Schm.) Eriks. & E. Henn., found that heating wheat leaves before inoculation temporarily increased susceptibility to both rusts, especially in such highly resistant varieties as Malakof (to *P. rubigo-vera* f. sp. *tritici*) and Heine's Kolben (to *P. glumarum*). Effects were most pronounced from 40 seconds in water at 50°C.

Materials and methods.—Lerma 52—one of the Kenya derivatives, a commercial wheat variety in Mexico—was the host plant used in these studies. This variety is highly resistant to wheat stem rust race 139A (*Puccinia graminis* Pers. var. *tritici* (Eriks. & E. Henn.) Shear, Ball, Jacks. & Stakman) at low temperature (65°F) and very susceptible at high temperature (85°F) (4, 5). It is one of the hosts that differentiates 139A from other subraces of race 139 (4).

Seedlings were planted in 4-in. pots and inoculated in the first-leaf stage. To obtain seedlings that were comparable in development, seed was planted at the lower temperature (65°F) two days earlier than at the high temperature (85°F). Leaves of seedlings grown at 85°F were darker in color than those grown at 70°F. Seedlings grown at both temperatures were divided into 2 groups inoculated at different temperatures (65 and 85°F). Inoculation techniques were those described by Stakman et al (6). The inoculated seedlings were kept in moist chambers for 24 hours at 65° or 85°F. Each group was then divided into two groups and transferred to the greenhouse benches of two adjoining sections of the greenhouse at an average of 74°F or 87°F.

Table 1.—Infection types produced and amount of infection centers produced on Lerma 52 wheat inoculated with race 139A of *Puccinia graminis* var. *tritici* and exposed to different temperatures at the pre-, during, and post-inoculation periods.

Treatment	Temperature (°F) at the time of Pre-inoculation	During inoculation	Post-inoculation	Infection types produced	Av. no. infection centers per leaf
1	70	65	74	0;	trace
2	70	85	74	0; & 1=*	5
3	85	85	74	X–	40
4	70	65	87	3 to 3++	50
5	70	85	87	3 to 4	10
6	85	85	87	3 to 4++	20

* Some flecks were about 2 mm in diameter.

Temperature was recorded every 6 minutes on a 12-point Brown Potentiometer thermograph. Thermocouples were placed at plant height on the greenhouse benches or inserted in the moist chambers with the inoculated seedlings. Temperatures at each location could be averaged for each hour, for each day and then for the 14 days of the experiment's duration.

Notes were recorded 14 days after inoculation, when the rust usually reached maximum development (Table 1; Fig. 1).

Experimental results.—*Effect on the amount of infection*—Seedlings in treatments 2, 3, 5, and 6 (Table

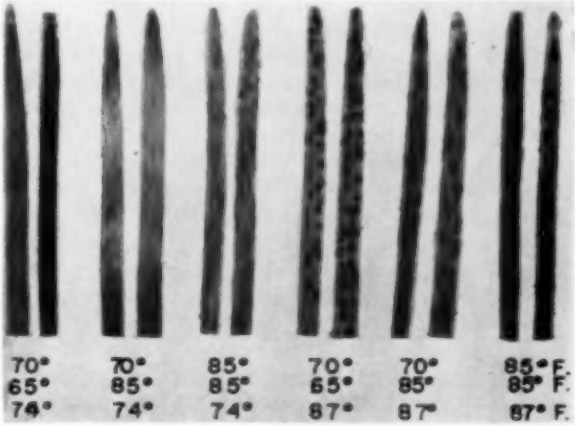

Fig. 1.—Reactions of seedlings of Lerma 52 wheat to race 139A of *Puccinia graminis* var. *tritici* at different pre-inoculation, inoculation, and post-inoculation temperatures (respectively, top to bottom).

1) were kept after inoculation for 24 hours at an average of 85°F. More infection centers per leaf developed on seedlings grown at 85°F (treatments 3 and 6) than on seedlings grown at 70°F (treatments 2 and 5) (Table 1).

Effect on rust reaction.—Comparing reactions of seedlings in treatments 2 and 3 and those in 5 and 6, differences were noted between the reaction of the seedlings in the two treatments in each of the two groups. Infection types 0; and 1= were produced on seedlings in treatment 2, and infection type X– on seedlings in treatment 3. The two treatments (2 and 3) differ only in the temperature before inoculation. Seedlings grown at the low temperature (70°F) and those grown at the high temperature (treatment 3) were inoculated together at 85°F and placed together at 74°F. The differences observed were obviously due solely to differences in pre-inoculation temperatures. The same trend was apparent for treatments 5 and 6, but the differences were not so pronounced.

Temperature in the moist chambers was also found to affect rust reaction. Slight differences existed between the reactions of seedlings in the moist chamber kept at high temperature and at low temperature. Necrotic flecks appeared on seedlings in treatment 1 (24 hours in moist chambers at 65°F), and infection types 0; and 1= on seedlings in treatment 2 (in moist chambers at 85°F). Also, infection types 3 to 3++ were noted on seedlings in treatment 4 and infection types 3 to 4 on seedlings in treatment 5. The difference in these treatments was only the temperature for 24 hours in the moist chambers (65°F or 85°F).

It was also noted that the reactions of seedlings of Lerma 52 inoculated with race 139A were sensitive to temperature following inoculation. The plants were resistant at 74°F (treatments 1 and 2) and susceptible at 87°F (treatments 4 and 5).

Discussion.—Post-inoculation temperature has been found to affect rust reaction, and its effect seemed to be quantitative (5). The longer the inoculated seedlings of certain wheats were kept at high temperatures, and the higher the temperature, the larger were the uredia. This was more pronounced with the temperature-sensitive wheat varieties (3, 5).

The results presented show that pre-inoculation temperatures affect the number of infection centers and rust reaction of Lerma 52 wheat inoculated with race 139A. More infection centers were produced and higher infection types were noted with a pre-inoculation temperature of 85°F than with 70°F. In general, high temperatures before inoculation increased the susceptibility of Lerma 52 to race 139A.

Temperatures before inoculation apparently had an effect on the constituents of Lerma 52 and influenced its rust reaction. This effect on constituents was noted in the color of the leaves of seedlings grown at the two temperatures. Seedlings grown at 85°F were darker than those grown at 70°F. Also, seedlings grown at high temperature (85°F) grew more rapidly than those grown at 70°F. The high temperature may favor production of the metabolic constituents of this variety in a form easy for this rust race to use.—Department of Plant Pathology and Botany, Institute of Agriculture, University of Minnesota, St. Paul 1.

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OVERWINTERING AND MIGRATION OF DITYLENCHUS DIPSACI IN ORGANIC SOILS OF SOUTHERN NEW YORK

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SUMMARY

Ditylenchus dipsaci overwinters in organic soils of southern New York, mainly as adults and preadult larvae. A few earlier-stage larvae were found, but no eggs. There was evidence of a partial downward migration in the fall and a partial upward migration in the spring. Mortality was high in the portion of the soil that became frozen in the winter. The species persisted in the soil in the absence of a susceptible crop for at least 2 years.

Literature review.—The taxonomy of the genus *Ditylenchus* is in a state of confusion. Many workers have shown the existence of biological strains. It has been suggested that *Ditylenchus dipsaci* (Kuhn 1857) Filipjev 1936 is not a single species, but rather a complex of taxa since there is some evidence that there are morphological differences among biological strains (11).

This nematode spends part of its life history in the soil, but probably does not reproduce readily outside of host plants. A carrot strain in England is reported to reproduce only in host plants (5). Strains in Dutch soils are thought to reproduce in weeds (9).

D. dipsaci has been observed to feed on soil fungi (16). It was not determined whether such feeding is common or of any importance in prolonging the existence of this nematode in the soil.

The longevity of *D. dipsaci* in soil lacking a host plant seems to vary with the soil and the strain of the nematode. A carrot strain in England in moist loam soil died out in less than 1½ years (5), but an oat strain in Scotland in moist soil lived up to 4 years (7). A strain attacking hyacinth and narcissus in Tasmania lived in soil for up to 3 years (12).

The period required for control also varies. Six years of lettuce, a nonhost crop, is reported to be necessary for control of a garlic strain in California (4). An onion strain in Australia requires rotations of up to 5 years (3). Rotations of 4–6 years are necessary for control of an oat strain in Scotland (7).

An onion strain in Australia is said to persist between onion crops in either soil or plant debris in soil (3). Chitwood, Newhall, and Clement, working with an onion strain in the organic soils of New York, were unable to find evidence that *D. dipsaci* overwintered in the soil (2). Those workers felt that this nematode persisted mainly in infected bulbs left in the fields.

The persistence of *D. dipsaci* in Dutch soils has been correlated with soil type (9), greater numbers being found in heavy soils than in light soils. In contrast, the numbers of other species of nematodes were often low in heavy soils and high in light soils.

Studies in Dutch soils also showed that this nematode is spread predominantly by migration in heavy soils and by cultivation in light soils (8). Infestations in heavy soils were roughly circular, and in light soils extended in the direction of cultivation.

The optimum temperature for movement of *D. dipsaci* in sand was reported by Wallace (13) to be 15–20°C, whereas Seinhorst (8) said this species in soil is less active at a high temperature (20°C) than at a low temperature (5°C).

D. dipsaci in sand has been shown to migrate for distances as great as 125 cm (10). It has been demonstrated that *D. dipsaci* can migrate through soil more rapidly than larvae of *Heterodera schachtii* Schmidt (13).

Methods.—For study of the overwintering of *D. dipsaci*, an infested field (field A) was selected in the summer of 1955. The soil is an organic soil containing some clay. A severely infested area was located so that soil samples could be taken throughout the winter. Samples were taken at intervals of about 3 weeks, starting in September.

Enough soil for a total of 1 quart was taken 1–12 in. deep at 7–10 locations within the selected area at each time of sampling. Soil temperature 6 in. deep was recorded at the time. The sample was mixed thoroughly and an aliquant of 100 cc was screened with screens of 20, 60, and 270 mesh. Nematodes from the 270-mesh screen were placed in watch glasses and the numbers of *D. dipsaci* (adults and larvae) determined.

After December 7, the samples were taken at two depths, 0–6 in. and 6–15 in., and soil temperatures were determined at 6 and 12 in. This method was chosen because it had been determined that the organic soils of southern New York usually freeze to a depth of about 6 in., and it was suspected that freezing might affect the survival of *D. dipsaci* in soil. The samples were examined in the same manner until June, 1956.

Then the method of extracting the nematodes was changed, though the samples were collected at 2 depths and mixed as before. Screening was discarded in favor of the centrifugal-flotation technique described by Caveness and Jensen (1). An aliquant of 50 cc of soil was taken from each sample. Comparison of the 2 methods indicated that centrifugal-flotation yielded about twice as many *D. dipsaci* as the screening method.

Samples were taken in field A from June, 1956, to September, 1957. Two more fields (B and C) were selected during the summer of 1956. Both fields contain soils with about the same amount of organic matter as in field A, but these soils are somewhat

Table 1.—Numbers of Ditylenchus dipsaci and temperatures at various depths in field A from September, 1955, to May, 1956.

Date	Soil temperature (°F) 6 in.	12 in	Numbers of *D. dipsaci*[a] 0–12 in.	0–6 in.	6–15 in.
Sept. 27, 1955	61		25+		
Oct. 25	46		25+		
Nov. 16	45		25+		
Dec. 7	36		25+		
Dec. 28	30	36		10	25+
Jan. 19, 1956	32	35		16	25+
Feb. 9	33	35		16	25+
Feb. 29	34	36		15	25
Mar. 21	35	37		12	19
Apr. 12	42	44		23	15
May 2	55	54		13	10
May 23	63	61		9	6

[a] Each number represents the *D. dipsaci* (adults and larvae) found by screening 100 cc of soil.

lighter in that the mineral fraction is mostly silt and sand. Infested areas were selected in each field, samples were taken from September, 1956, to September, 1957, in the same manner as in field A. The nematodes were extracted by the centrifugal-flotation method.

Field A had the following crops during the experimental period: 1955, onions; 1956, lettuce; 1957, lettuce. Fields B and C had onions in 1956 and lettuce in 1957.

Results.—Both adults and larvae of *D. dipsaci* were found in the soils of all 3 fields throughout the experimental period. Adults and preadult larvae were the forms commonly found. Smaller larvae were rare, and eggs were never found. In contrast, examination of infected onion tissue with the centrifugal-flotation technique revealed an abundance of all forms, including eggs. Gravid females were common in onion tissue but rare in soil.

The number of *D. dipsaci* in field A decreased from September, 1955, to May, 1956 (Table 1). No data on migration were obtained in the fall of 1955 because samples were taken only at 1 depth. The data obtained in the spring of 1956 show an increase in numbers in the upper soil layer and a decrease in the lower. This is interpreted as evidence of a partial upward migration.

The numbers of *D. dipsaci* found in field A from June, 1956, to September, 1957, indicate that the population fell to a very low level by the end of the first summer, during which an insusceptible crop (lettuce) was grown (Table 2). The population level during the second year, remained low but did not disappear, and there was no evidence of migration. Individuals of *D. dipsaci* found in the soil during the second year were usually preadult larvae and exhibited less physical activity than those found in onion tissue.

The numbers of *D. dipsaci* found in fields B and C from September, 1956, to September, 1957, indicate a partial downward migration in the fall, considerable mortality in the upper sampling region in the winter and a partial upward migration in the spring (Tables 3 and 4). The changes in the proportions of nematodes in the 2 regions is interpreted as evidence of partial migration, and the decrease in total number is interpreted as evidence of mortality. The populations dropped to a relatively low level during the summer in the absence of a susceptible crop. Preadult larvae were the form most common by September, 1957.

Discussion.—These data indicate that *D. dipsaci* overwinters in the organic soils of southern New York mainly as adults and preadult larvae. Smaller larvae appear of little importance, and eggs do not seem to

Table 2.—Numbers of Ditylenchus dipsaci and temperatures at various depths in field A from June, 1956, to September, 1957.

Date	Soil temperature (°F) 6 in.	12 in.	Numbers of *D. dipsaci*[a] 0–6 in.	6–15 in.
June 19, 1956	68	66	7	2
July 24	70	69	5	1
Aug. 27	72	71	3	1
Sept. 13	66	66	3	7
Sept. 26	60	59	4	4
Oct. 17	60	58	3	5
Nov. 8	52	52	1	1
Nov. 27	38	39	1	3
Dec. 19	35	37	0	2
Jan. 8, 1957	33	35	1	2
Jan. 30	34	34	2	1
Feb. 20	34	35	1	1
Mar. 13	42	40	0	0
Apr. 2	47	45	0	2
Apr. 23	57	55	0	0
May 13	69	64	0	1
June 3	65	63	0	1
July 1	72	71	1	0
July 29	74	73	1	0
Sept. 3	70	69	1	2

[a] Each number represents the *D. dipsaci* (adults and larvae) found in 50 cc of soil with the centrifugal-flotation technique.

Table 3.—Numbers of Ditylenchus dipsaci and temperatures at various depths in field B from September, 1956, to September, 1957.

Date	Soil temperature (°F) 6 in.	12 in.	Numbers of *D. dipsaci*[a] 0–6 in.	6–15 in.
Sept. 26, 1956	57	57	34	9
Oct. 17	65	62	39	8
Nov. 8	50	52	30	15
Nov. 27	35	38	18	23
Dec. 19	34	36	3	19
Jan. 8, 1957	33	35	4	15
Jan. 30	32	33	3	19
Feb. 20	34	34	3	15
Mar. 13	42	38	3	13
Apr. 2	49	45	9	10
Apr. 23	59	57	7	8
May 13	69	65	6	8
June 3	65	63	8	8
July 1	67	67	8	6
July 29	73	76	2	1
Sept. 3	70	69	3	6

[a] Each number represents the *D. dipsaci* (adults and larvae) found in 50 cc of soil with the centrifugal-flotation technique.

Table 4.—Numbers of Ditylenchus dipsaci and temperatures at various depths in field C from September, 1956, to September, 1957.

Date	Soil temperature (°F)		Numbers of *D. dipsaci*[a]	
	6 in.	12 in.	0–6 in.	6–15 in.
Sept. 26, 1956	57	57	27	7
Oct. 17	62	64	13	11
Nov. 8	50	52	12	14
Nov. 27	36	38	2	11
Dec. 19	34	36	1	12
Jan. 8, 1957	34	35	3	10
Jan. 30	32	33	2	16
Feb. 20	32	33	2	12
Mar. 13	42	38	12	6
Apr. 2	48	44	11	5
Apr. 23	62	60	8	3
May 13	72	67	5	2
June 3	66	65	2	3
July 1	70	69	4	2
July 29	75	76	1	5
Sept. 3	72	71	0	3

[a] Each number represents the *D. dipsaci* (adults and larvae) found in 50 cc of soil with the centrifugal-flotation technique.

be present. Thus, reproduction in the soil is at least quite rare. Adults and larvae are the stages toward which a chemical control program must be directed.

Onions left in the field do not appear to be an important source of overwintering nematodes, but sanitation should be encouraged since removal of infected bulbs at harvest will remove many nematodes before they move into the soil. Most onions left in the soil froze and decayed before spring. The nematodes in such bulbs must enter the soil or perish because *D. dipsaci* dies quickly in decaying onion tissue. Onions that survive the winter are usually small sprouts surrounded by numerous decaying scales. It is probable that most of the nematodes leave the onions in the fall, overwinter in the soil, and enter plants again in the spring.

Although *D. dipsaci* can still be present in the soil after 2 years of an insusceptible crop, the number may often be too low by the following spring for the establishment of infections in onions. The nematodes found by the end of the second summer appeared to be in a very low state of vigor. This species is not known to be parthenogenetic. Thus, it may be necessary for 2 nematodes of opposite sex to enter the same onion plant and meet within, since reproduction does not appear to occur in the soil. This situation is quite different from that presented by parthenogenetic species such as Meloidogyne which require only one female larva for the production of a new generation.

The high mortality of *D. dipsaci* in the first 6 in. of soil is probably due to freezing and thawing in this region. The survival rate would likely be higher during winters when less of the soil is frozen or freezing and thawing are less frequent. It is recognized, however, that the survival rate also might be low when this nematode must overwinter in the absence of host plants in a warm soil.

The majority of these nematodes are in the upper 6 in. of soil in the late summer and early fall following a crop of onions. The success of a soil fumigation might be determined in part by time of application. The nematodes are concentrated in a smaller layer before the downward migration. It would thus appear profitable to apply a soil fumigant at that time—late August and early September in southern New York. The onions are harvested in early August, about a month before soil temperatures normally begin to decrease appreciably. Spring fumigations are impractical there because the onions must be planted as soon as the soil can be worked.—Department of Plant Pathology, Cornell University, Ithaca, N. Y.

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THE EFFECT OF PYRIMIDINES AND 5-PHENYLAZOPYRIMIDINES ON THE MULTIPLICATION OF TOBACCO MOSAIC VIRUS

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Studies on the chemotherapy for plant virus diseases—V.

Contribution No. 42, Plant Pathology Laboratory, Faculty of Agriculture, Nagoya University.

SUMMARY

In a study of the inhibitory activity of pyrimidine and 5-phenylazopyrimidine derivatives on the multiplication of tobacco mosaic virus in excised tobacco leaf discs, 2,4,6-triamino-5-phenylazopyrimidine was significantly effective. The inhibition was not reversed by thymine, uracil, and cytosine. It is suggested that the mechanism of inhibition of virus multiplication by this compound is distinct from the inhibition of bacterial growth by the same compound.

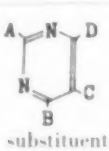
Among antiviral substances already reported, antimetabolites, such as purine and pyrimidine derivatives, show the most remarkable inhibition of plant virus multiplication. Following the discovery (7) of 8-azaguanine, a purine analog, pyrimidine analogs have attracted extensive attention. It has been found that the biosynthesis of tobacco mosaic virus (TMV) is inhibited by thiouracil (2), thiocytosine, and thiothymine (8). Schlegel and Rawlins (9) reported the inhibitory effect on TMV production of diazouracil, 5,6-diaminouracil sulfate, and 2-amino-4-methylpyrimidine. Davern and Bonner (3) showed that 5-fluorouracil (5-FU) inhibited virus production and that this inhibition was not reversed by thymidine or uracil.

Tanaka et al (11) synthesized many of the pyrimidine and 5-phenylazopyrimidine derivatives and made a detailed investigation of their inhibitory activity on the growth of *Lactobacillus casei* and *Tetrahymena geleii*, as well as on the multiplication of Yoshida sarcoma and Ehrlich ascites carcinoma. This paper reports the effect of these compounds on TMV multiplication.

Materials and methods.—All compounds tested were obtained from Takeda Pharmaceutical Industries, Ltd. These were dissolved in distilled water or, if insoluble in water, were dissolved in a small amount of ethanol or 2% $NaHCO_3$ and made up to desired volume with distilled water. The usual concentration of 150 μg/ml was lowered if it was insoluble or was toxic to leaf tissues. The strain of TMV used, the ordinary strain found in Japan, was supplied by the Tobacco Experimental Station. The test plant was usually *Nicotiana tabacum*, Turkish, but was sometimes the variety Bright Yellow (Table 2, Py-58–Py-83).

The compounds were tested as follows (4): 20 discs, 12 mm in diameter, cut from a detached tobacco half-leaf inoculated with TMV one day before, were floated on solutions of the chemicals in Petri dishes at 25°C under continuous illumination from fluorescent lamps. Controls were another 20 discs, from the opposite half-leaf and kept on distilled water. After 4 days the discs were removed, homogenized, and analyzed for TMV content by the method of Bancroft and Curtis (1).

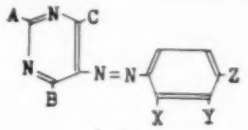
Table 1. Effect of pyrimidines on the multiplication of TMV.



Preparation no.	substituents A	B	C	D	Conc. of chemicals (μg/ml)	Changes in TMV multiplication*	Effect of compounds on leaves
Py-8	Cl	NH_2	NO_2	CH_3	10	−9 ± 7.0	none
Py-20	C_2H_5O	C_2H_5O	NO_2	$CH_3COCOOC_2H_5$	10	−13 ± 2.5	none
Py-22	–	OH	$NHCOCH_3$	CH_3	150	+6 ± 1.0	none
Py-23	NH_2	NH_2	–	NH_2	150	+4 ± 1.5	none
Py-24	NH_2	NH_2	NO	NH_2	75	+1 ± 0.9	slight yellowing
Py-25	NH_2	NH_2	NH_2	NH_2	150	−3 ± 6.0	none
Py-27	NH_2	CH_3	NH_2	CH_3	150	−1 ± 1.0	none
Py-28	NH_2	CH_3	$NHCOCH_3$	CH_3	150	−2 ± 4.1	none
Py-29	C_2H_5O	NH_2	NO_2	CH_3	75	−4 ± 2.0	none
Py-31	C_2H_5O	NH_2	NO_2	$CH_3COCOOC_2H_5$	20	−5 ± 2.0	none
Py-34	OH	OH	–	CH_2COOH	150	−19 ± 8.0	none
Py-38	NH_2	OH	–	NH_2	150	−18 ± 0.7	none
Py-45	NH_2	OH	NH_2	NH_2	150	−16 ± 0.7	none
Py-48	HS	OH	–	NH_2	150	−17 ± 2.0	none
Py-51	–	OH	NH_2	NH_2	150	−9 ± 0.7	none
Py-53	CH_3	OH	NH_2	CH_3	50	−26 ± 2.5	none
Py-76	NH_2	NH_2	–	CH_3	150	+6 ± 0.7	none

* Figures represent percentages of increase (+) or decrease (−) in TMV multiplication and average percentages of deviation from mean.

Table 2.—Effect of 5-phenylazopyrimidines on the multiplication of TMV.



Preparation no.	substituents A	B	C	X	Y	Z	Conc. of chemicals (μg/ml)	Changes in TMV multiplication[a]	Effect of Compounds on leaves
Py-26	NH_2	CH_3	CH_3	H	H	H	75	−7 ± 1.6	none
Py-37	OH	OH	CH_3	H	H	H	25	−6 ± 1.6	none
Py-40	NH_2	NH_2	OH	H	H	H	75	+9 ± 5.0	none
Py-41	NH_2	NH_2	NH_2	H	H	H	20	−55 ± 1.2	slight yellowing
							10	−48 ± 5.5	none
							5	−39 ± 9.5	none
							1	−17 ± 5.0	none
Py-58	OH	CH_3	CH_3	H	H	H	150	−10 ± 3.0	none
Py-64	NH_2	NH_2	NH_2	H	H	SO_3H	150	−14 ± 3.0	none
Py-68	NH_2	CH_3	CH_3	H	H	SO_3H	150	+1 ± 4.5	none
Py-71	NH_2	NH_2	OH	H	H	SO_3H	150	+6 ± 4.0	none
Py-74	NH_2	NH_2	NH_2	H	H	COOH	150	0 ± 0	slight yellowing
Py-78	NH_2	NH_2	CH_3	H	H	SO_3H	150	−2 ± 2.0	none
Py-79	NH_2	NH_2	OH	H	H	PO_3H_2	150	+8 ± 6.5	none
Py-80	NH_2	NH_2	NH_2	H	H	PO_3H_2	100	+6 ± 1.5	none
Py-81	NH_2	NH_2	NH_2	H	H	AsO_3H_2	150	−8 ± 1.5	none
Py-82	NH_2	NH_2	NH_2	SO_3H	H	H	150	−7 ± 2.5	none
Py-83	NH_2	NH_2	NH_2	H	PO_3H_2	H	150	+7 ± 5.0	none

[a] Figures represent percentages of increase (+) or decrease (−) in TMV multiplication and average percentages of deviation from mean.

Percentages of decrease in TMV multiplication were calculated by the ratio of optical density at 260 mμ of the treated to that of control.

Results.—Previous studies (10) have shown that, under the conditions of our experiments, maximum inhibition is 60–70%, accomplished by thiouracil. Most effective antibiotics, such as mitomycin C, actidione, and aureomycin hydrochloride, inhibited virus multiplication only about 30%. Thus, compounds showing 30% or more inhibition were considered comparatively promising.

None of the pyrimidines tested inhibited TMV multiplication effectively, though Py-53 (2,6-dimethyl-3-amino-4-hydroxypyrimidine) showed slight inhibitory activity (Table 1). Table 2 shows the inhibitory effect of 5-phenylazopyrimidine derivatives synthesized by introducing phenylazo radical into the nitroso radical of Py-24. Py-41 (2,4,6-triamino-5-phenylazopyrimidine) was significantly effective even at a fairly low concentration. Other compounds showed no inhibitory activity. For example, inhibitory activity was markedly reduced by substitution of the amino radical with methyl or hydroxy radical, or introduction of sulfonic acid, carboxylic acid, phosphoric acid, or arsenic acid into the benzene ring of the phenylazo radical.

The inhibition of virus multiplication by Py-41 was not reversed by thymine, uracil, or cytosine at concentrations greater than that of the Py-41 (Table 3).

Discussion.—Tanaka et al (11) and Kawashima (5, 6) reported that Py-41 inhibited the growth of *L. casei* and *T. geleii*, but that inhibition was reduced in compounds having substituents in the para-position of the benzene ring of the phenylazo radical. The latter compounds, in contrast—for example, those having substituents such as sulfonic acid, carboxylic acid, or phosphoric acid (Py-64, 74, 80)—inhibited the multiplication of Yoshida sarcoma and Ehrlich ascites carcinoma. Our experiments showed that Py-41 influenced TMV multiplication, but Py-64, 74, and 80 did not. It is therefore supposed that the compounds inhibiting the production of plant viruses are distinct from those showing an effective activity on tumors.

The antibacterial activity of Py-41 was reversed by thymine and leucovorin, but not by folic acid. It was therefore concluded that Py-41 inhibits the biosynthesis of the citrovorum factor from folic acid in bacteria (6). Davern and Bonner (3) showed that the inhibitory activity of 5-FU is not reversed by thymidine or uracil, and that the inhibition is not attributable to effects of the compound on the DNA metabolism of host plants, although, in bacteria, 5-FU can always act as an inhibitor of DNA synthesis. In the present experiments, the inhibitory activity of Py-41 on TMV

Table 3.—The action of thymine, uracil, and cytosine in reversing TMV inhibition by 2,4,6-triamino-5-phenylazopyrimidine (Py-41).

Compounds	Concentration (*M*)	Decrease in TMV multiplication[a]
Py-41	4×10^{-5}	−45 ± 5.0
Thymine[b]	1.6×10^{-4}	−45 ± 3.0
	1.6×10^{-3}	−43 ± 5.5
Uracil[b]	1.8×10^{-3}	−51 ± 7.0
Cytosine[b]	1.8×10^{-3}	−51 ± 6.0

[a] Figures represent percentages of decrease (−) in TMV multiplication and average percentages of deviation from mean.

[b] In addition, each solution contained Py-41 (4×10^{-5} *M*).

multiplication was not reversed by thymine, uracil, or cytosine. It may therefore be concluded that the mechanism of antimetabolite inhibition of TMV multiplication is not consistent with that of the inhibition of bacterial growth.—Plant Pathology Laboratory, Faculty of Agriculture, Nagoya University, Anzyo, Aichi-Ken (Japan).

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PURIFICATION BY DENSITY-GRADIENT CENTRIFUGATION, ELECTRON MICROSCOPY, AND PROPERTIES OF CYMBIDIUM MOSAIC VIRUS

M. K. Corbett

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SUMMARY

A disease charcaterized by sunken, reddish-brown necrotic streaks on the leaves of Cattleya orchids in Florida proved to be caused by Cymbidium mosaic virus (CyMV), as determined by host range, physical properties, and morphology. Three new nonorchidaceous hosts were discovered, all in the genus Cassia (*Cassia occidentalis*, *C. tora*, and *C. bicapsularis*). The virus induces local lesions in the 3 species 4–6 days after inoculation; no systemic infection occurred. *C. occidentalis* is a more satisfactory local-lesion test plant than are the other 2 Cassia species or previously reported hosts. The opposite leaflets of the evenly pinnate compound leaves are satisfactory for comparative inoculations. About 200–400 local lesions per inoculated leaflet were obtained with partially purified virus; with crude juice of diseased Cattleya leaves, lesion number was about inversely proportional to dilution. The virus, readily purified by rate zonal density-gradient centrifugation, occurred in the gradient column as a visible zone sufficiently pure for electron microscopy. Dissociation or aggregation of particles was very slight. The particles were sinuous rods 18 mμ wide, and over 60% were 480 mμ long.

Cymbidium mosaic virus (CyMV), first reported from Cymbidiums (11, 12), has since been found to be responsible for a disease of Cattleya orchids that is characterized by leaf necrosis (13, 16). Many species and hybrids of Cattleya and the closely related genera *Brassocattleya* and *Laeliocattleya* are susceptible (16). The virus has also been recovered from plants of *Epidendrum O'Brienianum* Rolfe, which exhibited brownish block spots and streaks on the underside of the leaves (14). The first nonorchidaceous host of the virus, *Datura stramonium* L., was reported by White and Goodchild (22). In this host the virus induced local lesions, without systemic infection, 13–14 days after inoculation. Other tested species of Datura were not susceptible (16). Hollings (10) and Murakishi (17) recently extended the host range of Cymbidium mosaic virus to include several other nonorchidaceous plants.

This paper reports on: purification of CyMV by rate zonal density-gradient centrifugation; a new local-lesion host for this virus; the physical and biological properties; and morphology of Cymbidium mosaic virus isolated from Cattleya orchids exhibiting leaf necrosis.

Symptomatology.—The diagnostic symptoms occur in the advanced stages of the disease. The leaves exhibit brownish sunken necrotic streaks. The internal leaf tissue is necrotic, and the epidermis, although not broken, collapses over these necrotic areas, imparting a sunken, rough, and furrowed appearance. The necrotic area is usually reddish-brown but may be black or red, depending on the orchid variety. The initial symptoms are small white streaks on the leaves; these enlarge and eventually turn brown. Fig. 1-A shows 3 leaves of Cattleya orchid with various degrees of leaf necrosis. The symptoms are more pronounced on the undersides of leaves. In severe cases the upper

leaf surface collapses, and entire areas of the leaf may be black and necrotic. These symptoms are similar to those described earlier (11, 12, 13, 16) except that chlorotic and necrotic ring patterns were rare during the investigation reported here. Attempts failed to recover virus from leaves with necrotic rings.

Flowers were generally smaller and fewer on diseased plants than on comparable healthy plants. No other flower symptoms were observed.

Virus isolates and methods of inoculation.—No satisfactory nonorchidaceous host was available for maintaining the virus. All experiments were conducted with inoculum from diseased Cattleya orchids collected in various orchid ranges in Florida. Cattleya leaves with symptoms similar to those in Fig. 1-A were ground in a food chopper, and the pulp was squeezed through cheesecloth. Expressed juice thus obtained was used for all experiments and inoculations. Unless otherwise stated, all inoculations were made by the gauze-pad method. Leaves to be inoculated were dusted with Carborundum, inoculated, and rinsed with tap water.

One isolate of CyMV, obtained from Cymbidium by D. D. Jensen, was used for comparative inoculations.

Cassia occidentalis as a local-lesion host.—Local lesions formed on the leaves of *C. occidentalis* L., *C. tora* L., and *C. bicapsularis* L. 4–6 days after inoculation. The lesions were small, discrete, brown spots that enlarged slightly (Fig. 1-B). Systemic infection did not occur. The lesions induced on inoculated plants of *C. tora* were black, and also enlarged slightly without systemic infection. *C. occidentalis* is more suitable for quantitative work than *C. tora* and *C. bicapsularis*, which have leaves that are smaller and more thigmotropic. Plants of *C. occidentalis* are easily grown from seed by seeding in vermiculite and transplanting to 4-in. pots of composted soil. Under Florida conditions the plants may attain considerable size and the opposite leaflets of the evenly pinnate compound leaves are adequate for comparative inoculations. With partially purified virus 200–400 lesions may be obtained on 1 leaflet.

A typical dilution curve with lesion number approximately inversely proportional to dilution was obtained when crude juice from diseased plants was used undiluted, and dilution with 0.01*M* neutral phosphate buffer as follows: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}. Each treatment occurred the same number of times in the same leaflet position. Forty-two leaflets were used for each treatment, and each experiment was replicated 3 times. Lesions were counted 6–10 days after inoculation. Average number of lesions per inoculated leaflet for each treatment were as follows: undiluted, 82; 10^{-1}, 125; 10^{-2}, 16; 10^{-3}, 3; 10^{-4}, 0.2; 10^{-5}, 0.

Anderson (1) reported that plants of *C. occidentalis* exhibited numerous local lesions without systemic infection when inoculated with the viruses of tomato ringspot, tobacco ringspot, or potato virus X. He previously found that plants inoculated with tobacco etch virus also produced local lesions, with or without systemic infection, depending on the strain of the virus used (personal communication). Those findings were verified in this study. In addition to these viruses, the lupine strain of bean yellow mosaic virus also induces local lesions, with systemic necrosis. Partially purified tobacco mosaic virus also induced minute local lesions, without systemic infection. Plants inoculated with alfalfa mosaic virus, 3 strains of potato virus Y, or 2 strains of cucumber mosaic virus exhibited no visible symptoms of infection, and the viruses could not be recovered.

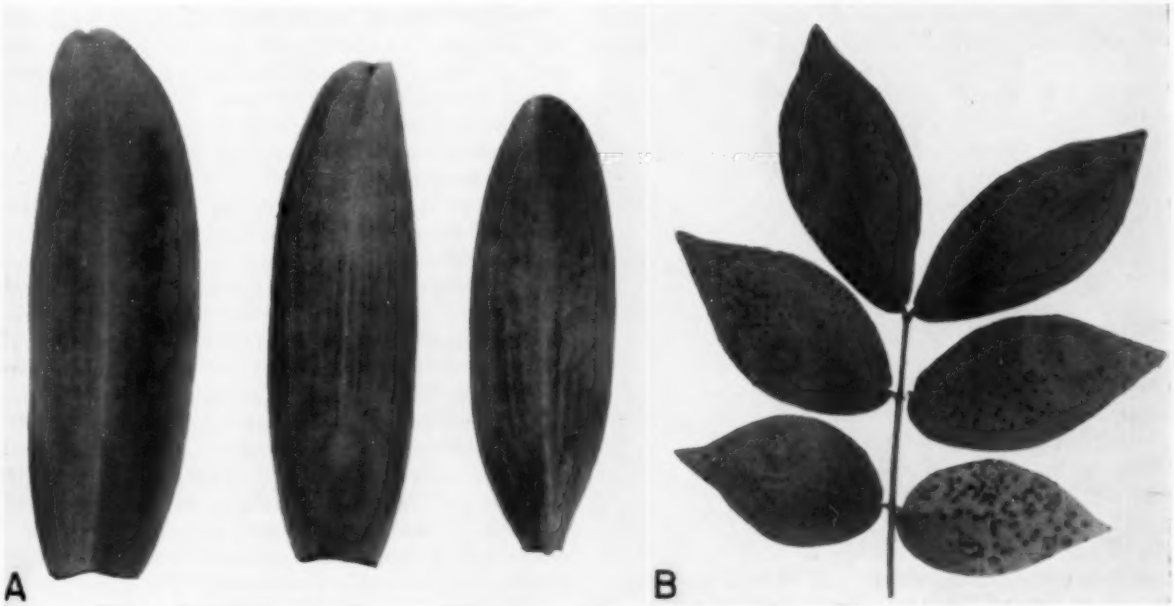

Fig. 1.—A) Necrosis on the lower surface of Cattleya orchid leaves resulting from infection by Cymbidium mosaic virus (×¼). B) Leaf of *Cassia occidentalis* exhibiting local lesions 7 days after inoculation with Cymbidium mosaic virus (×⅓).

The local-lesion hosts in the genus *Cassia* were discovered during the preliminary host-range studies, and plants of *C. occidentalis* were used for all recovery and index tests.

Host range.—Jensen (12) reported failure in attempts to transmit CyMV to 24 species of plants representing 20 genera of 15 families. Hollings (10) reported that plants of *Gomphrena globosa* L. exhibited irregular brown or reddish necrotic lesions when inoculated with CyMV. Murakishi (17) reported that all orchid virus isolates with which he worked induced local lesions in *D. stramonium* similar to those reported for CyMV (16, 22). He also reported that plants of *Nasturtium majus* [*Tropaeolum majus* L.], *Oryza sativa* L., *Passiflora edulis* Sims., and *Zinnia elegans* Jacq. were symptomless hosts of the virus.

Plants tested in the present work were inoculated when the primary leaves were fully expanded or when the first pair of true leaves was large enough. Ten plants of each species or variety were inoculated, and 2 plants of each were retained as uninoculated controls. Most tests were replicated during different seasons of the year. Attempts were made to recover the virus from all inoculated plants by inoculation to plants of *C. occidentalis*.

No infections were produced in 26 species (7 families) tested: *Vigna sinensis* (L.) Endl. var. Blackeye; *Lupinus albus* L., *Crotalaria spectabilis* Roth.; *Pisum sativum* L. vars. Alaska, Perfection, Little Marvel; *Phaseolus vulgaris* L. vars. Bountiful, Stringless Green Refugee; *Vicia faba* L.; *Phaseolus limensis* Macf. vars. Fordhook, Henderson Bush; *Datura meteloides* DC; *Capsicum annuum* L. var. California Wonder; *C. frutescens* L. var. Tabasco; *Solanum tuberosum* L. var. U.S.D.A. seedling 41956; *Lycopersicon esculentum* Mill. var. Marglobe; *Nicotiana tabacum* L. var. Turkish; *N. glutinosa* L.; *Tropaeolum majus* L.; *Cucumis sativus* L.; *Gomphrena globosa* L.; *Zinnia elegans* Jacq.; *Triticum aestivum* L. vars. Bledsoe, Costal; *Zea mays* L. vars. Dixie 18, Crockers c811A; *Sorghum vulgare* Pers. vars. Texas 601, Shallu; *Avena sativa* L. vars. Appler, Fulgrain; *Hordeum vulgare* L. var. Peru; *Oryza sativa* L. vars. Zenith, Caloro; *Secale cereale* L. vars. Florida Black, Gator; *Lolium multiflorum* Lam.

The experimental nonorchidaceous host-range of the Florida isolate was limited to 3 species of the genus *Cassia*, in the family Leguminosae, and to 1 species of the genus *Datura*, in the family Solanaceae. Inoculated plants of *Datura stramonium* L. produced local lesions, without systemic infection, 14–21 days after inoculation; this is in agreement with earlier reports (16, 22). Lesions occurred on the older leaves first and were not distinct enough for quantitative work. The older inoculated leaves often abscised before the lesions on the younger leaves were countable.

Some of the hosts reported by Hollings (10) and Murakishi (17) as susceptible to their isolates of CyMV proved insusceptible to the Florida isolate under the conditions used.

Local lesions induced by Jensen's isolate of CyMV from Cymbidium in plants of *C. occidentalis* and *D. stramonium* were identical to those induced in the same hosts by the Florida isolates.

The first symptoms observed when young Cattleya seedlings are inoculated with crude juice from diseased Cattleya plants are small, whitish streaks that eventually (5–8 weeks) develop into reddish-brown necrotic streaks. Ten seedlings were inoculated with expressed juice from leaflets of *C. occidentalis* exhibiting local lesions. Two of the seedlings developed symptoms of the disease 8 weeks after inoculation.

Physical properties.—To compare the physical properties of the Florida isolate with those reported in the literature for CyMV, the following tests were conducted. In all experiments, plants of *C. occidentalis* were used as the test plant and diseased Cattleya orchids for the virus source.

Dilution end-point was determined as previously described. The virus withstood dilutions of 10^{-4} but not 10^{-5}. These results are in agreement with those reported earlier (12, 17).

Thermal inactivation.—Two-ml samples of crude juice from diseased Cattleya orchids were heated for 10 minutes at different temperatures, cooled immediately in tap water, and used for inoculation. The inoculation scheme was the same as that for the dilution end-point experiments. Average numbers of lesions obtained with juice unheated and heated at various temperatures were as follows: unheated, 76; 60°C, 91; 62°, 18; 64°, 3; 66°, 0.5; 68°, 0.07; 70°, 0. Aging *in vitro* was at 20°C. A 20-ml sample of crude juice from diseased Cattleya orchids was tested daily for loss of infectivity. The experiment was ended in 14 days, when about 1 local lesion was obtained per inoculated leaf. These results compare favorably with those reported by Jensen (12) and Murakishi (17) for their isolates of CyMV.

Purification and electron microscopy.—The differential host-range of the viruses affecting orchids is limited. To obtain additional information on the identity of the Florida isolates, the virus particle associated with the diseased Cattleya orchids in Florida was compared morphologically with that reported for other virus-infected orchids.

Gold (7) reported the isolation of 2 types of virus particles from diseased orchids. Sinuous rods of 19×500 mμ were isolated from Cymbidium plants exhibiting mosaic. The other particle, occurring in various lengths, was 28 mμ in diameter. This particle was obtained from Cymbidiums and from Cattleya orchids exhibiting flower break. Gold and Jensen (8, 9) partially purified the virus of Cymbidium mosaic by differential ultracentrifugation and found the virus particles associated with the disease to be sinuous rods averaging 18×475 mμ. They found no comparable viruslike particles from healthy plants. Newton and Rosberg (19, 20) found 2 types of virus particle associated with a disease of Cattleya orchids: 1) a long, sinuous rod of 390×21 mμ and 2) a rigid rod of 300×24 mμ. They considered these particles as causal agents of a virus disease complex of Cattleya orchids. Jensen and Gold (16) partially purified a virus by dif-

ferential ultracentrifugation from both diseased Cattleya and Cymbidium orchids. From both they found a sinuous rod virus normally 448–488 mμ long. In several preparations from both sources they found no significant difference in particle length.

Since differential ultracentrifugation failed to purify the Florida isolate from diseased Cattleya orchids, density-gradient centrifugation similar to that reported by Brakke (3, 4) was used. Leaves of diseased Cattleya orchids were ground in a food chopper, and the juice expressed through cheesecloth. Single leaves from diseased or healthy plants were used for each experiment. The crude juice was diluted 1:1 v/v with buffer (0.01*M* KCl, 0.005*M* K_2HPO_4, 0.0005*M* KH_2PO_4; pH 7.7) and centrifuged 15 minutes in a Servall SP angle centrifuge at 3000 rpm (1280 G). Gradient columns for rate zonal centrifugation were made by Brakke's technique (5) as follows: 100, 200, 300, and 400 g of sucrose were dissolved in a liter of buffer and respectively layered in nitrocellulose centrifuge tubes at 4, 7, 7, and 7 ml. All gradients were stored at 10°C and made at least 1 day before use. A 2-ml sample of virus suspension (low-speed supernatant) was layered on top of the gradient and centrifuged 1.5 hours at 23,000 rpm (76,000 G) in the SW 25.1 rotor of a Spinco Model L preparative ultracentrifuge. Two tubes of each run contained material from diseased orchids. For comparison the third tube of each run contained material from healthy orchids.

After centrifugation the tubes were removed and examined in a dark room by shining a narrow beam of light from a Bausch and Lomb Nicholas illuminator into the tube from above and observing areas of light scattering. The tubes were punctured with a 1-in. 22-gauge hypodermic needle fitted to a syringe, and the visible zones were removed. The zones thus obtained were used for infectivity studies and electron microscopy. More than 1 visible zone was usual in each tube. The position of the zones was determined with a centimeter rule by measuring the region where the zone occurred in relation to the bottom of the meniscus. In the gradients for diseased material, a dense white zone 2–4 mm wide was usual about 5 mm below the meniscus; 1–1.5 cm below the meniscus was a bluish-white zone of about 4–5 mm. This zone did not occur in the gradients used with preparations from healthy orchids. The number and intensity of the zones varied, but in all experiments the zone that the diseased and healthy material did not have in common was in the same relative position, within 2 mm. After centrifugation the gradient columns were generally clear to slightly turbid, depending on the species or variety of the virus source. The juice from some orchids is very viscous and separation is not so definite. All centrifuged tubes contained a large green pellet.

The zones and all the areas above and below the zones were removed and tested without dialysis for infectivity on plants of *C. occidentalis*. The zone that the diseased and healthy did not have in common contained the greatest amount of virus as determined by local-lesion counts and electron microscopy. The area immediately above the zone gave an average of 0.045 local lesions per inoculated leaflet, compared with 179 for the zone. The area below the zone gave an average of 12 local lesions per inoculated leaflet. All samples were inoculated on 30 leaflets, and the experiment was replicated 15 times. The small amount of active virus above the zone may be attributed to a centrifugation time insufficient for sedimentation of all virus particles. The infectivity below the zone may be the result of virus aggregation.

The zones of 1 tube of each centrifugation were used for infectivity studies, and the zones of the other tube containing diseased material was used for electron microscopy. The zones of the tubes with material from healthy plants were used only for electron microscopy because the plants had been indexed for the virus before the experiment was initiated. The zones, and areas above and below, were removed as described and used directly, without dialysis for electron microscopy.

Collodion-filmed specimen screens were prepared, and the material to be examined was mixed with a small amount of polystyrene latex and sprayed on the screens according to the technique described by Backus and Williams (2). Some screens were washed with distilled water to remove sucrose, which would have interfered with resolution. The latex suspension (Dow Chemical Co., Midland, Michigan) was from run LS-057-A, with an average particle size of 264 mμ and a standard deviation of 6 mμ. Screens were shadowed with chromium at tan $^{-1}$ 0.31 and viewed with a Philips

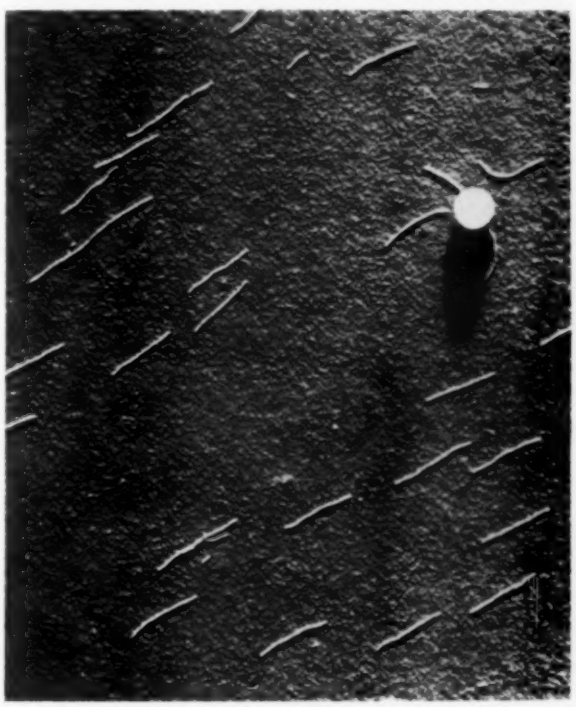

Fig. 2.—Particles of Cymbidium mosaic virus from visible zone obtained by rate zonal centrifugation shadowed with chromium at tan^{-1} 0.31 (26,000×). The polystyrene latex particle has a diameter of 264±6 mμ. (Photo by J. W. Carlisle.)

Fig. 3. Length distribution of Cymbidium mosaic virus particles obtained by rate zonal centrifugation.

electron microscope Model 100.

Fig. 2 shows part of a droplet pattern of a sample from the visible zone. The particle morphology compares very favorably with that reported for Cymbidium mosaic virus by Gold and Jensen (8) and Murakishi (18). The lengths are shown in Fig. 3. Fields were picked at random, and 500 particles were measured and plotted as a bar graph (Fig. 3). All particles were about 18 mμ wide. The most common particle length was 480 mμ. Of the 500 particles measured, 320 (64%) were that length. Comparable areas of the gradient columns used for healthy material were also viewed with the electron microscope. No rodlike particles were obtained from 15 leaves representing 15 healthy orchids.

Discussion.—The results show that the causal virus of the foliar necrosis disease of Cattleya orchids in Florida is Cymbidium mosaic virus. The disease in Florida is characterized by reddish-brown necrotic streaks, mainly on abaxial leaf surfaces. The necrotic and chlorotic rings illustrated by Jensen and Gold (16) are rare. This difference in symptoms may be attributed to virus strains, orchid varieties, and/or environmental conditions. The virus is common in Cattleya orchids in Florida, and is probably spread from plant to plant by mechanical means. The common practice of trading and selling orchid material is an ideal means of disseminating the virus. Lack of a satisfactory local-lesion assay plant has hindered qualitative and quantitative studies. *Datura stramonium* is not satisfactory for quantitative assay work, because lesions take about 2 weeks to develop, are indistinct, and develop on older leaves first. Murakishi (17) reported that the orchids *Spathoglottis plicata* Blume, *Miltonia* × *bluntii* Reichb. f., and *Arundina bambusifolia* Lindl., are susceptible to CyMV. The last two react with local lesions, but, though useful for certain studies, are more difficult to grow than *Cassia occidentalis*. The individual leaflets of the evenly pinnate compound leaves are large enough for comparative inoculations on opposite leaflets, and countable lesions develop on inoculated leaves 4–6 days after inoculation. The plant has made possible a rapid means of reliable indexing of suspected plants for the virus. Various orchids in Florida exhibiting smyptoms indicative of virus diseases have been indexed for CyMV. To date the local-lesion reaction has been specific for CyMV, which is responsible for the leaf necrosis of Cattleya orchid. The virus has been recovered from species of *Vanda*, *Rhynchostylis*, and *Phalaenopsis*. The stability of the virus and the procedures and cultural practices in orchid growing may help explain its wide occurrence in the family Orchidacea.

Gold and Jensen (8, 9, 16) reported a particle length of 475–480 mμ for their CyMV isolates from Cymbidium and Cattleya. Murakishi (18) reported similar measurements for isolates of the virus in Hawaii. Those reports and the results presented here support the hypothesis that particle morphology and normal length (6) are useful tools in virus identification, particularly when extensive comparative host-range studies are not possible.

Newton and Rosberg (19, 20) isolated rigid and sinuous rod virus particles from diseased Cattleya orchids. The symptoms they describe for the disease are quite different from those described here or reported for CyMV (12, 16) in Cattleya orchids. They reported no host-range studies, and it is difficult to determine the identity of the viruses causing the disease complex. Possibly the rigid rod was tobacco mosaic virus (21) or a mixture of CyMV and Odontoglossum ringspot virus. The latter is a 280×24-mμ rod (15) and can induce in Cattleya a mild flower break (14) similar to that reported by Newton and Rosberg (20).

Gold and Jensen (8) purified CyMV for electron microscopy by 2 or more cycles of differential ultracentrifugation. Purification by density-gradient centrifugation required only 1 centrifugation for 1.5 hours, and resulted in a final preparation relatively free of extraneous material and sufficiently pure for electron microscopy. Moreover, density-gradient centrifugation is a mild treatment, causing only slight particle dissociation or aggregation.—Plant Pathology Department, Agricultural Experiment Station, Gainesville, Florida.

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STREPTOMYCIN ABSORPTION, TRANSLOCATION, AND RETENTION IN HOPS

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SUMMARY

Bioassay methods were modified to determine streptomycin content of expressed hop plant sap. Streptomycin sulfate and nitrate preparations were applied to hop plants of different ages as foliar sprays and soil drench, and in lanolin paste. Streptomycin was readily absorbed from spray and paste, but not from soil. Absorption was greater and persisted longer in field-grown than in greenhouse-grown hops. Systemic concentrations were higher in young plants than in older plants. Peak concentrations were followed by sharp declines. Upward translocation was rapid; no downward translocation was detected. Minimum concentrations required in field hops were 12–19 ppm to reduce secondary leaf infection by the downy mildew fungus, and 46–69 ppm to enable shoots to recover from established systemic infection.

Introduction.—The downy mildew fungus, *Pseudoperonospora humuli* (Miy. & Tak.) G. W. Wils., which causes a severe disease of the hop (*Humulus lupulus* L.), has been a serious production factor in the hop industry for many years. During the primary cycle, in early spring, the disease is almost entirely systemic. Secondary cycles result chiefly in localized infection, though systemic infection also occurs (1). Reports of the systemic activity of streptomycin against fungi in plants (5) led to tests showing that streptomycin can control hop downy mildew in the field (4).

Preliminary investigations (6) indicated that streptomycin is readily absorbed from lanolin paste-streptomycin mixtures and readily translocated within hop plants. Further investigations were conducted to determine the internal concentration of streptomycin in various parts of treated plants. The measured concentrations in the various plant parts then indicated the pattern of absorption, translocation, retention, and breakdown. The minimum concentrations required to reduce secondary leaf infection and overcome established systemic infection were determined in the field.

Methods and materials.—*Bioassay procedure.*—Solutions of streptomycin in hop-juice diluent were pipetted onto a series of filter-paper disks. This technique was used both for solutions of known streptomycin concentrations as well as for juice samples. The filter-paper disks used were Schleicher and Schuell analytical grade No. 740E, of 12.5-mm diameter. Their maximum liquid absorption capacity is 0.1 ml. The saturated disks were placed on a ground-glass plate and dried 30 minutes at 37°C in an electric oven. Then 0.05 ml of a 1000-ppm mercuric chloride solution was applied to each disk to suppress the growth of contaminant organisms (7).

Hop samples were held in a deep freeze until all samples of an experiment had been collected. They were then thawed, washed 3 times in running tap water, and blotted dry, and a homogeneous portion was taken from the center for assay. This portion was placed in a Susi garlic press, and juice was expressed to impregnate a series of disks.

The test organism was a streptomycin-sensitive strain of *Bacillus subtilis* Cohn emend. Prazmolski

(ATCC 6633) maintained on potato-sucrose agar slants. Twenty-four ml of a freshly prepared Difco streptomycin-assay agar medium was dispensed into Petri plates, and 8 ml into test tubes. The plates were allowed to solidify, whereas the agar in tubes was held at 52°C in a water bath. The tubes were seeded by transferring a loopful of bacteria from a slant culture, and were agitated briefly. The mixture was then layered over the agar in plates and allowed to solidify at room temperature. The prepared assay disks were then placed 3 to each seeded plate, and the plates incubated 40 hours at 25°C. The zone of bacterial inhibition around the assay disks was measured with a pair of fine-point calipers and read against a vernier scale to tenths of a mm; the inhibition recorded was the mean width of the zone at 3 points equidistant on its circumference.

Streptomycin–B.-subtilis inhibition relationships.—In preliminary studies, a series of known streptomycin concentrations in hop juice were prepared for direct comparison each time samples were tested for streptomycin concentration (6). Later investigations employed a standard curve by which unknown streptomycin concentrations in assayed material were determined from measured bacterial inhibition.

The statistical validity of the standard curve was established in a series of 5 tests conducted on different dates. Active concentrations of 128, 64, 32, 16, 8, 4, 2, 1, and 0.5 ppm streptomycin were prepared in hop-juice dilutions, with hop juice alone as a control. Nine disks were used at each concentration in each trial, with conditions held as constant as possible on the different dates. The results, subjected to analysis of variance, showed no significant difference at the 5% level among dilutions of the streptomycin sulfate or nitrate preparations employed, or among replicates of a given concentration among the 5 different trials.

A standard curve was constructed on the means of the assay results, plotting streptomycin concentrations against bacterial inhibition. It became linear when the log of the streptomycin concentration was plotted against the mean value of bacterial inhibition. The regression of the distribution was then calculated, and the regression curve drawn (Fig. 1). The curve was later used for direct determination of streptomycin concentrations in assayed material. The concentration obtained represented the statistical mean expected for any given inhibition zone width. Herein the term "streptomycin concentration" means the concentration of streptomycin within the plants as determined by the assay methods described.

Preparation of hop samples for assay.—In greenhouse studies, cuttings of the variety Late Clusters were grown individually in 15-cm pots in a 16-hour-light day, with temperatures ranging from 16 to 29°C and relative humidities ranging from 54 to 70%. The plants ranged from 58 to 74 cm tall after 4–8 weeks. Test plants for each absorption trial were selected for uniformity. In the field, established hills of Late Clusters were used, and samples were taken from the basal growth, which consisted of shoots 15–38 cm long at treatment.

Streptomycin was applied in 3 media: lanolin paste mixtures, aqueous foliar spray with an adjuvant, and aqueous soil drench. Lanolin paste mixtures were prepared by blending 50 mg of active streptomycin into 1 g of anhydrous lanolin paste. One gram of this mixture was applied to each plant, providing a source point and a known available amount of antibiotic. Tap-water solutions of streptomycin at various concentrations were prepared, with glycerol added to make a 1% solution (2). In the greenhouse, sprays were applied with a hand sprayer until the foliage was thoroughly wet (about 20–25 ml per plant). In the field, applications were made with a power sprayer at about 70 psi nozzle pressure (about 100 ml per plant). Aqueous soil drench solution was prepared in tap water at 2000 ppm streptomycin and applied in 2 drenchings of 400 ml each on successive days.

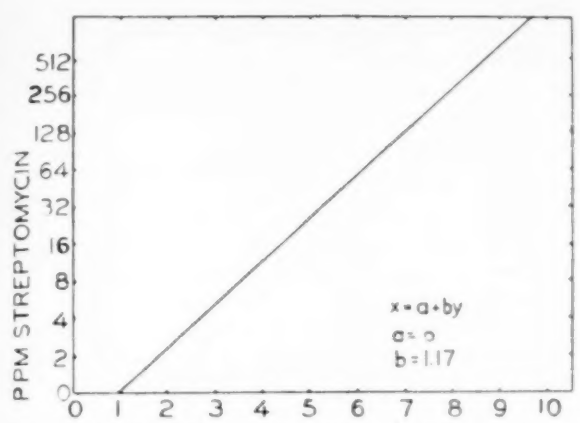
Experimental results.—*Studies with hops in the greenhouse.*—1) *Streptomycin absorption.*—Three replications of 3 plants each were sampled for streptomycin concentrations over a period of 72 hours after treatment with lanolin paste-streptomycin mixtures. The concentrations obtained, which reached respective maxima for the sulfate and nitrate formulations, of 57 ppm at 4 hours and 68 ppm at 2 hours, indicated rapid initial absorption of streptomycin from the lanolin paste mixtures. Concentrations declined sharply after attainment of maxima, which was interpreted as indicating rapid degradation or breakdown of the antibiotic.

Streptomycin absorption from foliar spray solutions and soil drench solutions was determined on samples taken from 3 replications of 3 6-week-old plants each over a 72-hour period. Peak streptomycin concentration was 23.4 ppm, at 8 hours, from the 500-ppm foliar spray but only 2.9 ppm, at 4 hours, from the soil drench. The pattern of streptomycin concentrations in plants treated with streptomycin sulfate is compared in Fig. 2 for lanolin paste mixture, foliar spray, and soil drench.

Four-week-old plants were treated with lanolin paste and foliar spray preparations of streptomycin sulfate, and samples were taken from 4 replications of single plants over a period of 192 hours. Rapid absorption was indicated by maxima of 152 ppm in 4 hours from lanolin paste and 242 ppm in 2 hours from 2000 ppm foliar sprays (Fig. 3). Absorption was more rapid from foliar spray, possibly because of the greater area of absorption. The decline in streptomycin concentration, almost as sharp as the increase, probably reflected a rapid breakdown of the systemic streptomycin.

The streptomycin concentrations obtained in plants are directly influenced by the concentrations of streptomycin in foliar sprays. Six-week-old plants sprayed with solutions of 500, 1000, and 2000 ppm streptomycin were sampled over a period of 10 days. Respective maximum streptomycin concentrations were 28.4 ppm, 81 ppm, and 152 ppm, all at 2 hours (Fig. 4).

The foregoing studies indicate that streptomycin is: 1) absorbed more rapidly by 4–5-week-old plants than



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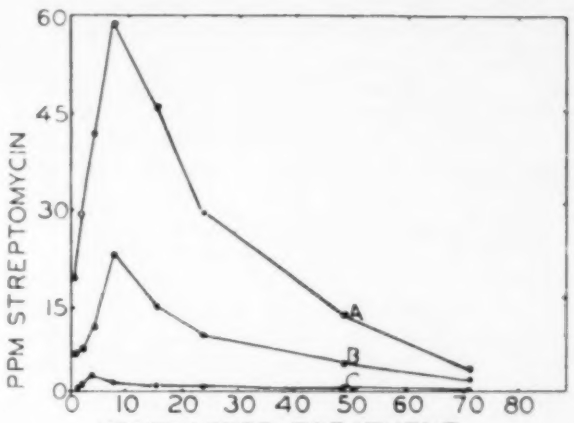

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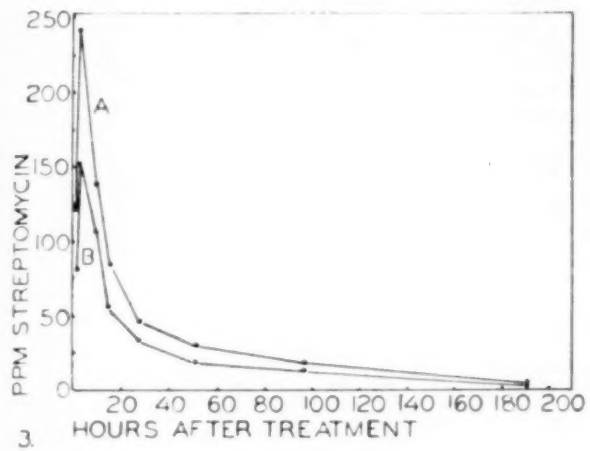

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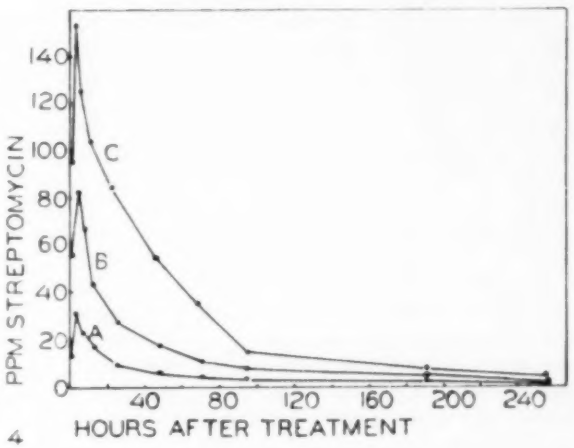

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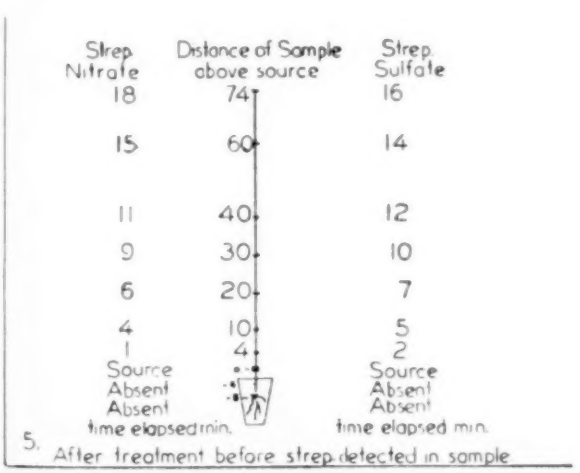

5. After treatment before strep. detected in sample

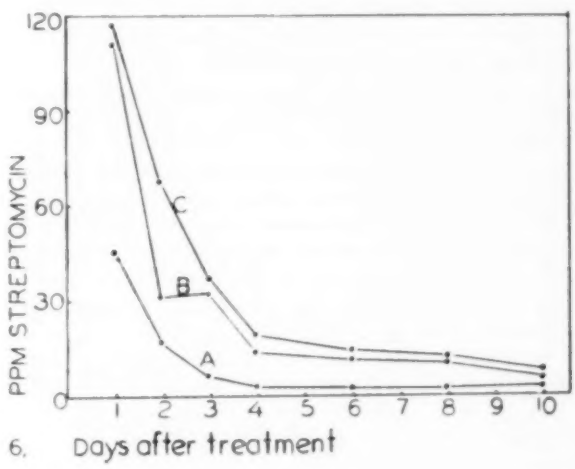

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Fig. 1–6.—1) Standard curve (streptomycin concentration to mean width of bacterial inhibition zones) from which streptomycin concentration in assayed samples was determined. 2) Streptomycin concentrations obtained in plants following A) lanolin paste, B) foliar spray, and C) soil drench applications of streptomycin sulfate. 3) Comparison of streptomycin concentrations resulting from A) 2000 ppm foliar spray and B) lanolin paste application. 4) Streptomycin concentrations resulting from foliar spray applications of A) 500 ppm, B) 1000 ppm, and C) 2000 ppm streptomycin. 5) Times elapsed after treatment before streptomycin concentrations measurable at various distances from the source; used in calculating translocation rates. 6) Streptomycin concentrations over a 10-day period following A) one 1000-ppm foliar spray, B) two 1000-ppm foliar sprays 21 days apart, and C) two 1000-ppm streptomycin foliar sprays 16 days apart.

Table 1.—Efficiency of streptomycin absorption by hops from lanolin paste and foliar spray applications of streptomycin sulfate.

Application method	Moisture content (g)	Maximum conc. (ppm)	Systemic strep. (mg.)	Absorption efficiency (%)
Lanolin paste[a]	16.1	131	2.09	4.15
Foliar spray[b]	14.9	81	1.21	6.06

[a] 50 mg streptomycin sulfate applied per plant.
[b] 20 mg streptomycin sulfate applied per plant.

by 7–8-week-old plants; 2) absorbed more readily from foliar spray than from lanolin paste; 3) absorbed more rapidly from nitrate preparations than from sulfate, though total amounts absorbed are no greater; 4) absorbed in direct proportion to the concentrations in foliar spray; and 5) broken down or utilized rapidly following attainment of maximum concentrations.

The efficiency of streptomycin absorption from lanolin paste and foliar spray applications was determined with 4 replications of 5-week-old plants. Samples were taken at 4-hour intervals beginning 2 hours after the treatment, and the maximum streptomycin concentrations were obtained. The portions of the samples from which 1.0 ml of juice per sample was expressed for assay were returned to the remainder of the samples. Green weight was determined, and the samples were dried to constant weight at 118°C in an electric oven to determine the moisture content of the plants. The amount of streptomycin in the plants was obtained by multiplying the mean moisture content of the plant by the mean peak concentration of the antibiotic. From the known amount of streptomycin applied to the plants, the efficiency of absorption was calculated to be 4.15% for lanolin paste application, and 6.06% for foliar spray treatment (Table 1).

2) *Streptomycin translocation.*—Rapid upward translocation was suggested by maximum concentrations of streptomycin attained after treatment with lanolin paste mixtures applied to the first node of the stems. Plants were sampled over a 72-hour period in 3 portions above the point of application: 0–20, 20–40, and 40–60 cm. A rapid absorption with attendant high peak concentration in the lowest sample (142 ppm at 8 hours) was followed by later peaks in the middle and top samples (68 ppm at 24 hours), with maxima of a lower magnitude (54 ppm at 24 hours) in the intervening portions.

A series of plants treated with lanolin paste mixtures of streptomycin applied to the first node of stems were sampled at 7 positions over a 72-hour period. Leaf samples taken at 4, 10, 21, 30, 42, and 62 cm above the point of streptomycin application yielded maximum concentrations that diminished with distance from the source and required successively longer periods to attain. Maximum concentrations were reached in the tips (74 cm above the streptomycin source) sooner than in the intervening leaf samples, and the peak attained was greater except for the 3 leaf samples closest to the streptomycin source (Table 2). These results indicate translocation of streptomycin in greater quantities to the growing tip than to the intermediate leaves. The progressively lower maximum streptomycin concentrations at higher positions excepting the tips suggests rapid degradation of the streptomycin in the plants or selective accumulation of the antibiotic in various parts.

The rate of streptomycin translocation in hop plants was determined with 90 6-week-old plants from which couplet leaves from 3 plants at each position were taken for each sample. Samples were taken from 7 positions at 1-minute intervals over a period of 30 minutes after treatment with streptomycin in lanolin paste. The translocation rate of streptomycin in the plants was calculated by dividing the distance of a sample from the streptomycin source by the elapsed time after treatment before streptomycin concentration (0.4 ppm) was measurable in the sample. The rates of streptomycin translocation from the source to the growing tips were respectively found to be 4.0 cm per minute and 4.6 cm per minute for streptomycin absorbed from the nitrate and sulfate preparations. Elapsed time before detection of streptomycin at each position is given in Fig. 5 for both preparations.

Attempts to determine downward translocation of streptomycin in hops over a period of 30 minutes were unsuccessful in stem and root samples taken 4 and 8 cm below the streptomycin source. Similarly, no measurable streptomycin concentration could be detected in the basal portions of 4 hop plants to which a 1000-ppm foliar spray had been applied on the terminal one-third.

3) *Retention of streptomycin.*—In the first of 2 studies to determine the persistence of streptomycin in hops, streptomycin sulfate and nitrate were applied in lanolin paste to 4 replications of 5-week-old plants, and samples were collected over a period of 20 days. Mean streptomycin concentrations attained were: 3.5 ppm at 3 days; 1.9 ppm at 7 days; 1.4 ppm at 10 days; 1.2 ppm at 13 days; 0.8 ppm at 16 days; and none detectable at 20 days. In the second experiment, with 7-week-old plants given a 1000-ppm streptomycin foliar spray, mean concentrations were: 5.8 ppm at 3 days; 2.9 ppm at 7 days; 2.0 ppm at 10 days; 1.7 ppm at 13 days; 1.0 ppm at 16 days; and only a trace at 20 days.

The persistence or retention of streptomycin by hops

Table 2.—Time required and maximum concentrations of streptomycin in leaf samples at various distances above the source of streptomycin sulfate and nitrate applied in lanolin paste to hop stems.

Height above source (cm)	Streptomycin sulfate		Streptomycin nitrate	
	Time (hr)	Conc. (ppm)	Time (hr)	Conc. (ppm)
4	4	142	2	133
10	8	83	8	69
21	20	69	20	58
30	24	30	24	36
42	30	17	30	18
62	30	12	30	12
74[a]	16	36	16	28

[a] Sample consisted of growing tips.

Table 3.—Degree of secondary leaf infection by hop downy mildew and systemic streptomycin concentrations in hops at daily intervals after 2 applications of 1000 ppm streptomycin foliar spray 16 days apart.

Interval to inoculation (days)	Leaf infection, Hoerner rating		Streptomycin (ppm)
	Treated	Check	
1	1.22*	3.58	98
2	1.28*	4.30	61
3	1.72*	4.19	41
4	1.89*	4.39	25
5	2.50*	4.22	22
6	2.80*	4.14	19
7	4.22	4.20	12
LSD at 5%	0.86		

*Significantly (5% level) less infected than simultaneously inoculated check.

is characterized by 3 stages: 1) rapid initial absorption and fairly high concentrations; 2) sharp declines closely following maximum concentration, probably due to breakdown; and 3) a gradual decline of the concentration of streptomycin over a period of 2 weeks or more.

Studies with hops in the field.—1) *Pattern of streptomycin concentrations following foliar application.*—Field-grown hops were treated in a large-scale experiment with a foliar spray consisting of a 1000 ppm streptomycin sulfate solution. Plant samples were taken at intervals for 10 days in order to investigate the pattern of distribution of the streptomycin concentration in the various plant parts. Basal shoots of the treated plants were 15–38 cm long, and additional samples were taken from this growth. The maximum concentration of streptomycin attained was 45 ppm. This occurred in 2 days (Fig. 6-A). In a similar experiment in the greenhouse, the maximum concentration attained was 81 ppm. It is apparent that absorption by the field-grown plants is slower than that by the plants grown in the greenhouse. The disappearance of streptomycin in the field-grown plants was apparently offset by the greater amount of the chemical available as indicated by the persistence of the antibiotic in the plants.

Plants receiving a second treatment of 1000 ppm streptomycin spray, 21 days following the first application, exhibited a striking increase in the maximum concentrations during a 10-day sampling period following treatment. In this instance, the maximum concentration attained was 109 ppm, and the levels of concentration of streptomycin were correspondingly higher over the sampling period (Fig. 6-B). A further trial was conducted to study the concentration levels of streptomycin attained in plants receiving 2 spray applications of streptomycin 21 days apart. Plants with vines 7–10 ft. tall received 2 applications of 1000 ppm streptomycin solution as a spray, 16 days apart. Samples, taken over a 10-day period, yielded a maximum concentration of 116 ppm, with an over-all pattern (Fig. 6-C) quite similar to that described above (Fig. 6-B).

2) *Determination of effective streptomycin concentrations.*—An experiment was conducted in a Late Clusters yard to determine the minimum streptomycin concentration required to reduce secondary infection of hop foliage by the downy mildew fungus. The tests involved 2 blocks of hops, each with an equal number of plants. The first block received 2 applications of a 1000-ppm streptomycin spray applied to the foliage, with a 16-day interval between sprays, and the second block was untreated. Plots of 3 hills in each block were inoculated daily after the second application, the inoculum consisting of a sporangial suspension sprayed onto the underside of the leaves. Samples of 36 leaves per plot were taken 9 days after each plot had been inoculated, and the samples from both treated and untreated plots were rated by the Hoerner (3) rating scale for severity of secondary leaf infection. Samples were collected from plants in the treated block to determine the streptomycin concentrations at inoculation. Analysis of variance of leaf infection severity showed that significantly less infection occurred in sprayed plants inoculated up to 6 days after the second spray treatment than in untreated check plants inoculated at the same time (Table 3). Some infection occurred at each inoculation date, but some of it was undoubtedly due to natural infection existing before inoculation.

The streptomycin concentrations were 19 ppm at 6 days and 12 ppm at 7 days, indicating that the minimum concentration of streptomycin in hops required for significant reduction of secondary leaf infection lies between 12 and 19 ppm.

A second trial, conducted to determine the streptomycin concentration required to arrest the growth of established systemic downy mildew mycelium, consisted of 3 replications of 5 plants, each possessing 1 systemically infected shoot at treatment. Each plant was sprayed with a single foliar spray at concentrations ranging from 500 to 2000 ppm streptomycin. The infected shoots were individually tagged and observed daily for 16 days. The condition of infected shoots at 16 days was recorded as dead, recovered, or remaining infected. Samples were taken over a period of 8 days to determine the maximum streptomycin concentrations resulting from each concentration in the sprays. The maximum concentrations attained and the condi-

Table 4.—Maximum systemic streptomycin concentrations reached after foliar spray applications of streptomycin at 6 concentrations, and condition of infected shoots 15 days after streptomycin treatment.

Concentration applied (ppm)	Concentration attained (ppm)	Hop shoot numbers		
		Dead	Recovered	Infected
500	26	2	0	13
750	46	1	0	14
1000	69	9*	2	4*
1250	80	8*	5	2*
1500	96	9*	6	0*
2000	136	3*	11	1*
Check		3	0	12

*Significantly (at 5% level) different from check at the 5% level.

tion of systemically infected shoots following each treatment are presented in Table 4.

As determined by subjecting the data to analysis of variance at the 5% level of significance, sprays of 1000, 1250, 1500, and 2000 ppm active streptomycin resulted in significantly less infection than in untreated checks, but sprays of 500 and 750 ppm did not. The maximum streptomycin concentrations reached were 46 ppm and 69 ppm, respectively following sprays of 750 and 1000 ppm streptomycin. The minimum streptomycin concentration required in plants to arrest established downy mildew infection was between 46 and 69 ppm.

Based on the streptomycin concentrations required, concentrations were effective for 1–3 days after 2 applications of 1000 ppm foliar spray in the field (Fig. 6-B,C).—Department of Botany and Plant Pathology, Oregon State College, Corvallis.

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CONTROL OF VASCULAR WILT DISEASES OF CARNATION BY CULTURE-INDEXING

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SUMMARY

The effectiveness of Hellmers' Broth Method for detecting carnation cuttings infected with *Fusarium oxysporum* f. *dianthi* and *Pseudomonas caryophylli* was determined by culture-indexing tip cuttings from plants growing in commercial greenhouses in New York, Pennsylvania, and Colorado, establishing apparently pathogen-free cuttings as single units in commercial nucleus blocks, and observing for symptoms for 14 months. About 40% of the original plants from which cuttings were taken showed symptoms of the 2 diseases. Of the 3564 cuttings indexed, 0.4% yielded the pathogens. Of the 2121 cuttings established as single units in nucleus blocks, 10 exhibited vascular wilt symptoms during the 14-month period. Three (0.14%) of these yielded *F. oxysporum* f. *dianthi*, 4 (0.19%) *P. caryophylli*, and 3 no pathogen. It is concluded that careful selection will eliminate the major portion of infected cuttings, that Hellmers' Broth Method did not allow for 100% detection of the 2 pathogens in infected cuttings not eliminated by selection, and that the establishment of selected or culture-indexed plants as single units in nucleus blocks is essential for pathogen-free production of this floricultural crop.

The vascular wilt diseases of the perpetual flowering carnation (*Dianthus caryophyllus* Linn.), incited by *Fusarium oxysporum* Schlecht. f. *dianthi* (Prill. & Del.) Snyd. & Hans. and *Pseudomonas caryophylli* Burk., have for many years caused serious economic losses to carnation growers of the United States (5, 12). Control has been difficult because these pathogens may infect the roots and colonize the vascular system of carnation without symptom expression (22, p. 70; 37, 38). Thus, with this vegetatively propagated plant, the pathogens may be disseminated within the cutting and the diseases increased without the knowledge of the propagator.

Since the original description of Fusarium wilt (27, 30) and of bacterial wilt (7, 25), investigators have sought for methods of eliminating the pathogens from commercial carnation varieties and sources of resistance in commercially acceptable varieties or clones. No commercially acceptable carnation varieties are known to be resistant to either pathogen. In fact, most of the Sim varieties, the major portion of commercially grown carnations today, are quite susceptible to both organisms.

Chemotherapeutic control of Fusarium wilt has been reported (6, 16, 33), but this method is little used by commercial carnation growers. Control of bacterial wilt with systemic chemical treatments and antibiotics has also been reported (1, 4, 17, 22), but commercial control has not been satisfactory, and these materials are not widely used by commercial growers. That approach, however, still holds promise.

Culture-indexing of vegetative cuttings has provided a means of detecting the pathogens and eliminating infected cuttings. In combination with a sanitation program to prevent reintroduction of the pathogens into pathogen-free plants, culture-indexing is the most effective method of controlling these diseases. The present study was conducted to determine the effectiveness of a recently described method (20, 21, 22) of detecting and eliminating carnation cuttings infected with the pathogens causing Fusarium and bacterial wilts.

Review of literature.—Various methods of culture-indexing have been described, often in publications not readily available to plant pathologists. The major contributions are therefore reviewed here.

Indexing for control of an ornamental plant disease was perhaps first suggested by Mangin (27), in 1899.

for the control of Fusarium wilt of carnation. Since the mycelium of the causal fungus grows systemically in the stem and is transmitted in outwardly healthy vegetative cuttings he detected infection by immersing the basal 2–3 cm of each cutting in water at 15°C for 24 hours. Cuttings showing a white growth were destroyed; the others were removed and immediately dipped into a solution containing 15 g/L Napthol B and 45 g/L soap to kill spores on the surface.

Culture-indexing was not again suggested for control of a plant disease for over 40 years; in 1941 Dimock (8) recommended it for control of Verticillium wilt of chrysanthemum. He first outlined and then elaborated on (9, 10, 13) what is now known as the Plate Method of culture-indexing, involving potato-dextrose agar in Petri dishes as a medium to detect *Verticillium albo-atrum* Reinke & Berth. in small tissue slices from the surface-sterilized base of chrysanthemum cuttings. Recent reports (20, 21, 22) crediting Guba (19) with introducing this method in 1952 are thus in error.

Suggestions and descriptions for using this method to detect *F. oxysporum* f. *dianthi* and *P. caryophylli* in carnation cuttings were reported by a number of workers (11, 15, 19, 28, 29, 39, 40). The method is described in detail by Forsberg (15), Tilford (39), and Guba (19). These workers all suggested or used the Plate Method.

In 1954, Tammen (34) described a method for culturing carnations that involved wrapping the surface-sterilized cutting in a sterile cotton plug and placing the plug in a sterile vial containing Barnes' medium (31), modified to enhance bacterial growth, so that the basal end of the cutting is immersed in the medium. The vials with cuttings were placed in a humidity chamber for 5–6 days, and then inspected for fungus or bacterial growth. All cuttings with such growth were discarded. Use of this method in producing pathogen-free carnations has been further discussed by Baker (2). A variation has been reported by Shurtleff and Sherwood (32).

Hellmers (22) found that the Plate Method did not always detect all plants infected with *P. caryophylli*, especially those with only a few infected vessels. Other workers (36) have reported similar findings and the authors have observed that a liquid medium, such as nutrient broth, is more sensitive for the detection of bacterial infections than the previously used agar slants or plates.

The most recent method of culturing carnation cuttings is the Broth Method, described and elaborated by Hellmers (20, 21, 22), and followed by further work by others (23, 24, 36). In this method small sections are cut from the surface-sterilized base of a carnation cutting, placed in a test tube containing a broth medium, and incubated at a temperature near optimum for growth of the pathogens. In a modification by Glasscock (18) the broth is replaced by a solid agar slant, and the cut tissue pieces are placed on the surface of this slant. The variation does not differ essentially from a current recommendation for detecting *V. albo-atrum* in chrysanthemum cuttings (14).

Materials and methods.—Carnation tip cuttings were collected from commercial greenhouses in New York, Pennsylvania, and Colorado. In New York, cuttings were collected from September to March from plants with no obvious symptoms of either wilt disease. During the preceding summer, about 95% of the plants in the greenhouses had shown symptoms of one or both of the vascular wilt diseases. In fact, losses had been so high that the grower was forced to replant some areas twice to get a partial stand. In January, 25% of the Pennsylvania plants from which cuttings were collected showed the basal canker symptom of bacterial wilt (35) though none showed recognized symptoms of Fusarium wilt. Cuttings were collected from these plants in early spring. Cuttings collected in Colorado were from plants thought to be pathogen-free. Cuttings in each state were cultured in a laboratory in that state in lots of 300–500 during the period of collection.

In each case culture-indexing was done as follows: Selected cuttings were 1 internode longer than those usually used for propagation. Each cutting was placed in a test tube containing 15 ml of 1:9 solution of commercial Clorox (5.25% sodium hypochlorite) plus 0.10–0.25% Dreft (sodium lauryl sulfate) so that the lower internode of the cutting was covered. After 5 minutes, the cutting was removed from the test tube and placed on absorbent paper toweling. Sterilization of toweling was discontinued when freshly opened packs were found to give no more contamination than sterilized towels. The basal ½ in. of the cutting was cut off with a previously sterilized razor blade and discarded. Then 2–4 thin slices of the stem were cut from the basal portion of the cutting (Fig. 1-A,G) and placed in a numbered test tube containing previously sterilized Bacto-Nutrient Broth plus 1.5% glucose (Fig. 1-E,I). The test tubes were incubated at room temperature or at 28–30°C, conditions suitable for growth of both pathogens.

Though most infected cuttings can be detected in 4–5 days, a few may not be detected until 10–14 days. Tubes containing only sterilized broth were incubated with the others to serve as checks, and all tubes were compared with these when readings were made.

Readings were based on the clearness of broth in the tubes. If the broth became cloudy or filled with mycelium (Fig. 1-J), the cutting was eliminated (Fig. 1-H). If it remained clear and there was no evidence of microbial growth (Fig. 1-F), the cutting was retained (Fig. 1-B) and considered to be free of the 2 vascular wilt organisms. All positive cultures were retained for identification of the bacterium or fungus. Fusaria were identified as to species, and all other fungi as to genus. Bacterial cultures were compared with agar-slant cultures of *P. caryophylli* on potato-dextrose agar; cultures not identical to these were classified "unknown."

Each cultured cutting was placed in a tube of sterile distilled water (Fig. 1-B,H), numbered to correspond with the broth culture, and stored at 3°C until final readings were made. Cuttings considered patho-

gen-free were then rooted in a rooting medium previously steam-treated (180°F for 30 minutes). Rebreaking the cutting (Fig. 1-C,D) was necessary because the sodium-hypochlorite–detergent treatment injured the tissue and retarded or prevented rooting.

The rooted cultured cuttings were established in 6- to 8-in. pots of steam-treated soil. Individual pots were placed on concrete blocks on the greenhouse floor or widely spaced on a raised bench in an isolated area of a commercial greenhouse. Thus, each plant was established as an independent unit, and the block was entirely separate from commercial carnation production. A plant developing symptoms of a vascular wilt disease was removed for laboratory culture and diagnosis. This block of plants actually formed the nucleus of the mother block propagation system previously described (36).

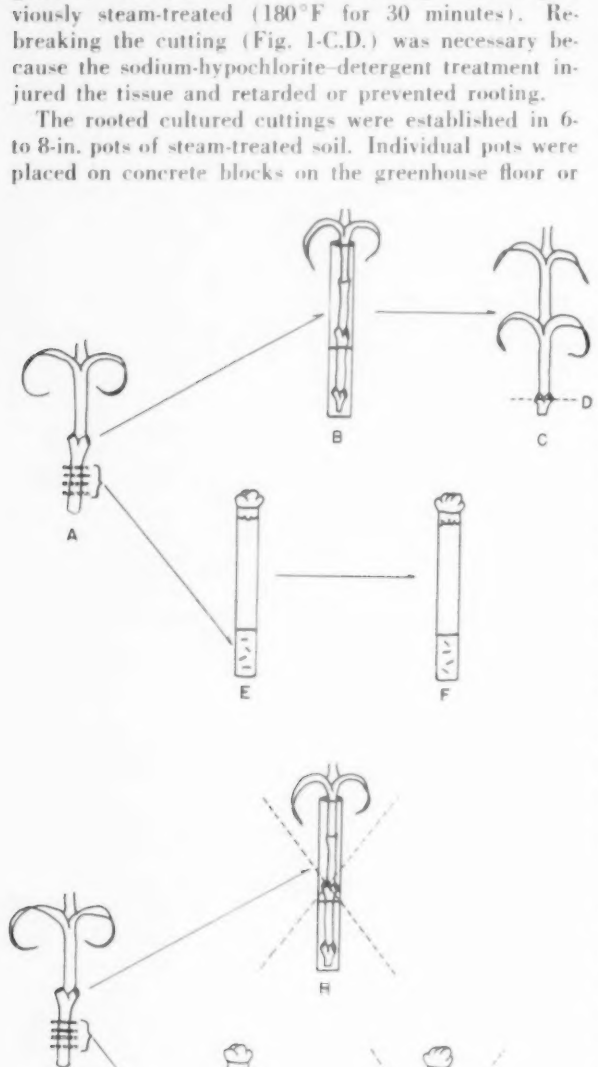

Fig. 1.—Diagram showing the steps in culture-indexing carnation cuttings: **A–F**) when the cutting is pathogen-free; **G–J**) when the cutting is infected with 1 or more of the vascular wilt pathogens. **A,G**) Sectioning lower internode of cuttings. **E,I**) Test tubes containing sterile nutrient into which the thin sections are placed and incubated for 10–14 days. **F**) Thin sections in broth, showing no evidence of microbial activity; correspondingly numbered cutting B is retained for rooting. **J**) Thin sections in broth showing evidence of microbial activity; correspondingly numbered cutting H is discarded. **B,H**) Culture-indexed cuttings stored in tubes of sterile distilled water at 3°C until final readings are made on broth cultures. **C**) Pathogen-free cutting being prepared for rooting. The cutting must be rebroken at D because the sodium-hypochlorite–detergent treatment injures tissue and reduces rooting.

Results.—Of 3564 cuttings thus culture-indexed, 11 were infected with *P. caryophylli* and 3 with *F. oxysporum* f. *dianthi*. In addition, 86 cultures yielded bacteria resembling *Erwinia* sp., an organism recently reported to incite a slow wilt disease of carnation (26), but pathogenicity tests with representative isolates were negative. Three cultures yielded fungi similar to *Phialophora* sp. Pathogenicity tests with 2 of them were negative; the third was not tested. Unidentified bacteria, considered contaminants, were encountered in 10–20% of the cultures. Contaminant fungi (*Penicillium* spp., *Chaetomium* spp., *Hormodendrum* spp., and others) were encountered in 5–10% of the cultures. Other fungi of possible pathogenic significance were *Alternaria*, *Phoma*, and *Phomopsis* sp., and *Rhizoctonia solani* Kuehn.

The nucleus block of 2121 plants was observed for 14 months for the 2 vascular wilt diseases. Of the 10 that developed possible symptoms, 3 yielded *F. oxysporum* f. *dianthi*, 4 yielded *P. caryophylli*, and 3 yielded organisms of unknown identity. Sixteen plants developed symptoms similar to those described as induced by *Erwinia* sp. in carnation (26). Bacteria isolated from them were negative in pathogenicity tests.

Discussion.—Losses from contaminants during culturing varied from 10 to 90% in individual lots of 300–500 cuttings, but averaged 20–40% for all cuttings cultured. The amount of contamination present is a direct result of the manner in which the cuttings are handled before being cultured. Losses from contamination are smallest when cuttings are taken from plants and cultured immediately. Any excess handling or storage may result in excessive losses. Losses ran as high as 90% with one group of unrooted cuttings shipped in moist sphagnum moss from Denver, Colorado, to Wantagh, New York. Jenkins (24) urged immediate culturing of cuttings, since incidence of bacterial contaminants is often very high when cuttings are stored in polyethylene bags, in which condensation is frequent and humidity high.

The results raise 2 question: 1) why were so few infected cuttings detected by culture-indexing, and 2) why did a small percentage of infected cuttings pass through the culture-indexing process undetected?

The answer to the first question could involve one or more of 3 factors: 1) the time of the year the cuttings were selected for culturing, 2) the distribution of the pathogens within the host at the time the cuttings were selected, and 3) the careful selection of cuttings used for culturing. In New York the percentage of infected plants in the greenhouses from which cuttings were selected was 95%, as indicated by symptoms

during the previous summer, the period of maximum symptom expression. In Pennsylvania the percentage of infected plants was 25%, as indicated by symptoms during January. The cuttings used for culture-indexing, however, were not taken during the summer but were carefully selected during the fall, winter, and early spring, when the wilt organisms are more or less inactive and when symptom expression is at a minimum. In addition, these were tip cuttings selected at a height of 12–18 in. In infected carnation plants that survive the summer and in plants infected in late summer or early fall, the Fusarium wilt organism may be confined during the following winter to the lower 2–6 in. of the plant (3), and it is possible that the bacterial wilt pathogen acts similarly. Unpublished data by the senior author indicate that in certain varieties of carnation, especially the Sim varieties, *F. oxysporum* f. *dianthi* does not colonize shoots before symptoms are obvious. In addition preliminary results indicate that cuttings infected by *P. caryophylli* can be obtained from infected plants before symptoms appear. It thus seems very probable that many of the pathogen-free cuttings came from infected plants. Careful selection of cuttings is obviously very important. Apparently, careful selection of tip cuttings alone will eliminate many infected cuttings before culture-indexing.

As to the second question, no data bear on failure of the method to eliminate infected cuttings completely. Maybe none were missed, but recontamination occurred. This seems unlikely in view of the many precautions. It is more likely that some of the cuttings were undetected by culture-indexing. Culture-indexing for fungi in this way depends on spread of the pathogen within the host only as a continuous growth of hyphae up the stem (10). It is possible, however, that spores produced within the plant are carried in the vascular stream beyond the growth of the hyphae, as suggested by Wickens (41) from studies on Phialophora wilt of carnation (*Phialophora cinerescens* (Wr.) van Beyma), and by Dimock (10) from work on *V. albo-atrum* in chrysanthemum. The bacterial pathogen probably does not follow a pattern of continuous growth in the host stem, and thus it is possible that single cells or clumps may pass through an internode without infecting it. If so, a negative culture from such an internode would not guarantee that the cutting would be pathogen-free above the point of culture. Lelliot (26) suggested that such does occur with *E. chrysanthemi*.

Of the plants in the nucleus block established by the methods discussed here, only 0.3% were infected. This small percentage would be a constant danger to the cutting producer, especially if the propagator became careless or if environmental conditions favored spread and development of the diseases. The importance of the nucleus block in maintaining pathogen-free stock cannot be too strongly emphasized. Maintenance of plants as individual units in the nucleus block enables the propagator to detect, before large numbers of cuttings are taken, any infected plants that passed through the culture-indexing process. Since the recommended procedure for maintenance of pathogen-free carnation propagating stock (36) requires that the nucleus block be renewed each year, the yearly culturing involved should gradually eliminate the known vascular wilt organisms and also detect any new organisms introduced. —Department of Plant Pathology, Cornell University, Ornamentals Research Laboratory, Farmingdale, Long Island, N. Y., and Departments of Botany and Plant Pathology, Pennsylvania State University, University Park, and Colorado State University, Fort Collins.

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BACTERIAL SPOT OF TOMATO AS INFLUENCED BY TEMPERATURE AND BY AGE AND NUTRITION OF THE HOST

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SUMMARY

Tomato plants showed marked differences in susceptibility to bacterial spot on aging; the older the leaves, the lower the susceptibility. The disease progressed through 4 fairly distinct phases: 1) lesions water-soaked and enlarged to 1–2 mm; 2) lesions turned brown; 3) chlorosis appeared and spread; 4) leaves dried, turned brown and leathery, and drooped. The disease developed most rapidly at a continuous 24°C, the temperature favoring good vegetative growth of the host plant. Next most favorable were 24° and 28° night temperatures regardless of day temperature. A night temperature of 16° suppressed the disease, regardless of day temperature. When N was varied, disease index was highest at 50 ppm, and declined steadily with increase in N, the difference becoming significant at 400, 800, and 1600 ppm. When P was varied the disease indices were high and very close to one another at 16–512 ppm; those at 4 and 1024 ppm were significantly lower. When K was varied, the disease indices were not consistently different from 12.5 to 800 ppm; at 1600 the index was markedly reduced. Disease suppression at extremely high levels of N, P, and K appeared partly due to high osmotic value of the nutrient; balanced solutions at similar osmotic values gave similar reductions in disease index.

Bacterial spot of tomato, *Lycopersicon esculentum* Mill., incited by *Xanthomonas vesicatoria* (Doidge) Dowson continues to be an important disease in the eastern United States. Chief damage is to fruits that become infected when immature, but leaf blight may be important when the disease becomes epidemic early in the growing season. The present investigation is concerned with development of the disease on leaves. Particular emphasis was placed on the relation of age and nutrition of the host and of air temperature to the progress and severity of leaf symptoms.

Materials and methods.—Used throughout were isolate 0-2894 of *X. vesicatoria*, secured from the State Plant Board of Florida, and the tomato variety Bonny Best. A stock culture of the organism was maintained on PDA (potato-dextrose agar) at 4°C. Tomato seedlings were raised in vermiculite until the first true leaf developed. Unless otherwise stated they were selected for uniformity and transplanted to 4-in. clay pots containing greenhouse compost soil. Plants were grown at 26±2°C and 12-hour daylight. Daylight was supplemented by 200-watt lamps. Inoculations were made *in situ*, without a moist chamber. A suspension of the bacteria was sprayed onto the lower surfaces of the leaves with a DeVilbiss atomizer attached to a compressed-air line. Inoculum consisted of a 36–48-hour culture grown on PDA in Petri dishes at 28° and washed into the requisite amount of sterile distilled water.

In leaves most susceptible at inoculation the disease progressed through 4 fairly distinct stages, briefly described as follows: 1) The lesions remained water-soaked for about 3 days, gradually enlarged, and extended from the lower to the upper surface of the leaflet. 2) The lesions turned brown in the center, with a narrow pale green halo sometimes visible on the upper surface of the leaflet; this stage lasted 2–3 days.

Table 1.—Relation of tomato plant age at inoculation with Xanthomonas vesicatoria to development of bacterial spot.

Leaf position[b]	Disease indices[a] of leaves inoculated at leaf-stage indicated								
	4	5	6	7	8	9	16	17	19
1	[c]	25	0	0	0	25	0	0	0
2	[c]	25	25	25	25	25	0	0	0
3	100	25	25	25	25	25	25	0	25
4	100	50	25	25	25	50	25	0	25
5		25	75	75	25	50	25	25	25
6			75	75	100	100	25	25	25
7				50	100	100	25	25	25
8					50	100	50	25	50
9						100	50	50	75
10							50	50	75
11							75	50	75
12							75	75	75
13							100	100	75
14							100	100	100
15							100	100	100
16							100	100	100
17								75	100
18									100
19									75

[a] 0 = no signs; 100 = 100 or more lesions per leaflet, extreme chlorosis and/or brown areas, conspicuous epinasty of leaflets and/or leaves.

[b] Leaves on each plant assayed were numbered upward, beginning with the lowest leaf.

[c] Data not taken.

3) The tissue between lesions became chlorotic, and leaflets and leaves showed decided epinasty; this stage lasted 2–3 days. 4) Infected leaves turned brown, became dry and leathery, and drooped.

If several leaves had expanded when plants were inoculated, leaves of various ages responded differently in number, size, and type of lesion. Scale of disease ratings was as follows: 0 = no signs of disease; 25 = lesions green, water-soaked, translucent, or just beginning to turn brown but with no chlorosis, and fewer than 50 lesions per leaflet; 50 = 50–100 lesions per leaflet, brown, and with very small chlorotic halos; 75 = 100 or more lesions per leaflet, brown, and conspicuous chlorosis around and between lesions; 100 = 100 or more lesions per leaflet, extensive chlorosis, affected portions may have turned brown and dried out, pronounced epinasty of leaflets and/or leaves. The disease index for any given treatment was derived by averaging the class numbers of plants in the treatment.

Experimental results.—*Age of leaf.*—When a plant with 7 expanded leaves was inoculated *in situ*, only a few lesions developed on the lower 4 leaves. They remained small and pink to gray, and if they extended to the upper surface of the leaf they remained as brown specks within a chlorotic halo 2–3 mm in diameter. The lesions on the younger leaves were numerous and progressed through the 4 disease stages described above.

Table 1 presents data on development of the disease of leaves of plants grown to different ages before inoculation. The stage of growth at inoculation was expressed in terms of the number of leaves expanded on the plant. Leaves were numbered beginning at the base of the plant, and the youngest leaf counted was about 1 in. long. The plants of various ages were inoculated simultaneously, and the final readings were recorded 12 or 13 days later. In most cases the lowest leaf was immune to infection after the plants had reached the 6-leaf stage. In plants at the 5-leaf stage or greater there was a progressive increase in susceptibility to full disease development up to the youngest or the next-to-youngest leaf. In the oldest plants inoculated the 2 oldest leaves were immune, and only the 14th to the 18th were susceptible enough to be placed in the highest disease class. Davis and Halmos (3) noted a similar relation of age to susceptibility in 6-leaf tomato plants.

Relation of temperature.—The effect of different continuous and alternating day-night temperature treatments on the disease was studied during the winter of 1957–58. Plants were grown in 4-in. pots, as described earlier, for about 3 weeks until the 7-leaf stage was reached. Plants of uniform size were selected, inoculated, and transferred to 16, 20, 24, and 28°C constant-temperature greenhouses. Some plants were held at the respective continuous temperatures; others were alternated between various day-and-night temperatures. The plants during the postinoculation period were subject to natural winter daylight. With alternating day-and-night temperatures the respective periods were about 10 and 14 hours. Four plants were used per treatment, and an index of disease development was recorded for the fifth and sixth leaves of each plant 12 or 13 days after inoculation.

Table 2 presents data at 4 continuous temperatures. There was a progressive increase in disease index from 16° to 24°, and a decline at 28°. Under the particular winter light conditions that prevailed, 24° was the optimum continuous temperature for the development of disease in susceptible leaves.

In Table 3, data from plants at both continuous and alternating day-and-night temperatures are arranged, for convenience in comparison, in 4 groups according to day temperature in the left index column and according to night temperature in the right index column. When plants at a given day temperature were exposed to the 4 respective night temperatures there was in most cases an increase in disease index with

Table 2.—The relation of continuous air temperature to development of bacterial spot on tomato leaves.

Temp.[a] (°C)	Disease index[b] in 3 experiments			
	1	2	3	Av. index
16	25	25	50	33
20	75	50	50	58
24	100	100	100	100
28	75	50	100	75

[a] Plants held at temperatures mentioned after inoculation for 12 or 13 days.

[b] Each value is the average of indices of the fifth and sixth leaf of 4 plants recorded 12 to 13 days after inoculation. 0 = no signs, 100 = 100 or more lesions per leaflet, extreme chlorosis and/or brown areas, conspicuous epinasty of leaflets and/or leaves.

Table 3.—The relation of various continuous and alternating day-and-night temperatures to development of bacterial spot on tomato leaves.

Temp. °C[a]		Disease	Temp. °C[a]		Disease
Day	Night	index[b]	Night	Day	index[b]
16°	16°	38	16°	16°	38
	20°	25		20°	25
	24°	75		24°	25
	28°	50		28°	25
20°	16°	25	20°	16°	25
	20°	50		20°	50
	24°	50		24°	63
	28°	75		28°	75
24°	16°	25	24°	16°	75
	20°	50		20°	50
	24°	100		24°	100
	28°	75		28°	88
28°	16°	25	28°	16°	50
	20°	75		20°	75
	24°	88		24°	75
	28°	75		28°	75

[a] Plants held at temperatures indicated after inoculation for 12 or 13 days.

[b] Each value is the average of indices of the fifth and sixth leaf of 4 plants in each of two experiments recorded 12 or 13 days after inoculation. 0 = no signs, 100 = 100 or more lesions per leaflet, extreme chlorosis and/or brown areas, conspicuous epinasty of leaflets and/or leaves.

increase in night temperature up to 24°C. Except at 20° day temperature, the disease index declined at 28° night temperature. When the same data are grouped at the respective night temperatures it is obvious that 16° night temperature suppressed the disease more consistently than did 16° day temperature.

Relation of host nutrition.—The continuous-drip equipment and quartz sand culture described by Gallegly and Walker (5) were used in preliminary experiments. In later experiments, with more levels desired of N, P, and K than were possible in the drip system, the porcelain crocks had Pyrex glass wool placed against the drainage holes. Tomato seedlings grown in vermiculite for about 12 days and showing the first true leaf were selected for uniformity. Six plants were transplanted into each pot, and nutrition treatments were applied. Two weeks later the plants were thinned to 4 per pot. The basal nutrient solution, 100 ppm N, 15.5 ppm P, and 115 ppm K, was modified to give solutions high or low in any one of the 3 elements. Where the osmotic value of a solution was less than 1 atmosphere it was adjusted to 1 with sodium chloride. The nutrient treatments were applied every 5 to 7 days at the rate of 1 liter per pot. Three plants per pot were inoculated 20–24 days after being transplanted. The disease index for the fifth and sixth leaves of each plant was recorded 12 or 13 days later, at which time the fresh weights of the diseased and healthy plants were recorded. The results with various levels of N, P, and K are presented in Table 4.

In preliminary experiments with continuous-drip equipment the plants grown at 5 ppm N were slender, stunted, and chlorotic, and showed purpling of stems and veins. The few small lesions that developed were chiefly on the younger leaves, and remained water-soaked for an abnormally long period. In later experiments, plants at 1600 ppm N were stunted and darker green than normal. The few lesions that developed on such plants remained very small, turned brown or gray in 2–3 days, and progressed no farther. Growth was definitely reduced at 25 ppm and 800 ppm N, but with 50–400 ppm there was little difference in the fresh weight of plants at the end of the experiment. In any given treatment, diseased plants weighed slightly less than the healthy plants. The highest disease index was consistently at 50 ppm N, with an abrupt drop above 400 ppm.

With phosphorous at 4 ppm and 1024 ppm the plants were abnormally dark green and showed definite depression in plant weight. Purpling of stems and veins was conspicuous in low-P plants. There was a moderate reduction in growth at 512 ppm. In any given treatment, diseased plants weighed slightly less than healthy plants. Disease index was consistently highest at 64

Table 4.—Development of bacterial spot on leaves of tomato plants grown in sand culture with various levels of nitrogen, phosphorous, and potassium.

Nutrient levels (ppm)			Disease index[a] in 3 experiments			Av. index	Av. fresh wt. (g) of plants	
N	P	K	1	2	3		H[b]	D[c]
25	15.5	115	88	50	46	61	22	20
50	15.5	115	92	54	67	71	37	29
100	15.5	115[d]	71	42	54	58	43	36
200	15.5	115	63	46	58	56	45	38
400	15.5	115	54	38	54	49	41	34
800	15.5	115	46	29	38	38	32	26
1600	15.5	115	25		25	25	15	12
100	4	115		46	58	52	24	20
100	8	115		50	71	61	38	29
100	16	115		54	88	71	40	36
100	32	115		46	96	71	41	35
100	64	115		63	96	80	42	35
100	128	115		58	92	75	43	35
100	256	115		63	83	73	41	35
100	512	115			79	79	30	28
100	1024	115			50	50	17	17
100	15.5	12.5			79	79	32	22
100	15.5	25		54	83	69	35	39
100	15.5	50		46	71	59	34	33
100	15.5	100		38	67	58	34	36
100	15.5	200		46	83	65	39	34
100	15.5	400		75	79	72	38	35
100	15.5	800		29	88	59	37	32
100	15.5	1600		25	33	29	32	28
LSD	19:1					19		
	99:1					25		

[a] Each value is the average of indices of the fifth and sixth leaf of 6 plants recorded 12 or 13 days after inoculation. 0 = no signs, 100 = 100 or more lesions per leaflet, extreme chlorosis and/or brown areas, conspicuous epinasty of leaflets and/or leaves.

[b] The values are averages of 2 healthy plants in each treatment and experiment.

[c] The values are averages of 6 diseased plants in each treatment and experiment.

[d] Basal nutrient.

ppm, and about equal at 8 to 512 ppm. Indices were significantly lower at 4 and 1024 ppm than at 15.5 ppm, the level of P in the basal nutrient.

With potassium, the differences in fresh weight of plants at the end of experiments were slight between 25 ppm and 1600 ppm. In most treatments diseased plants weighed slightly less than healthy plants. At 400 ppm and above the plants were darker green, and slightly stunted at 1600 ppm. At 5 ppm (in the continuous-drip system) they were stunted, the lowest leaves were chlorotic, and other leaves were rough in texture with the margins curled downward. Large discolored water-soaked lesions developed before and/or after inoculation on stems and petioles of such plants. The reaction was suggestive of low-K tobacco plants described by Allington and Johnson (1) and Johnson (7), and that of cucumber plants described by Van Gundy and Walker (12). The significantly lower indices in plants at 1600 ppm were the only consistent change in disease brought about by changes in K level.

In the experiments described, reductions in disease index were generally most significant at very high concentrations of N, P, and K. At those levels, however, the theoretical osmotic value of the nutrient solutions ranged from 2 atmospheres (1024 ppm P) to 5.7 atmospheres (1600 ppm N). To determine whether such high osmotic values of an otherwise balanced nutrient solution would similarly affect plant growth and disease development, experiments were conducted with high osmotic values secured by adding the required amount of sodium chloride. Both the basal solution (0.5H) and a solution with twice the concentration of salts (1H), with desired modifications in osmotic value, served as controls for evaluation of the other treatments. The results are presented in Table 5.

The greatest retarding influence on disease development was obtained in 1H and 0.5H treatments with an osmotic value of 5.7 atmospheres, paralleled by 1600 ppm N. The effect of 1600 ppm K was paralleled by 1H and 0.5H treatments with an osmotic value of 2.4 atmospheres. The average fresh weights of healthy and infected plants subjected to different treatments show that 1H and 0.5H gave about the same amount of growth, and that fresh weight was decreased by either a marked decrease or a marked increase in the concentration of any one of the elements or an increase in osmotic value. The plants grown at high osmotic concentration or high element concentration were stunted and dark green, and the leaves sometimes appeared flaccid in bright weather, favoring high transpiration.

Discussion.—Factors like light, temperature, host nutrition, and age of the host plant condition its physiology and, consequently, its reaction to a pathogen. Postinoculation temperature treatments had profound influence on the development of bacterial spot. About 30°C was reported as the optimum temperature for growth of the bacterium in culture and for disease development (4, 6). A day temperature of 23–26° and a night temperature of 17–20° were reported as the optima for good vegetative growth and fruiting of tomato (13, 14). At continuous higher temperatures, fruiting was suppressed and vegetative growth was enhanced. In the present investigation, a continuous 24° was most favorable for disease, whereas night temperatures of 28° and/or 24° were the next most favorable, regardless of day temperature. At continuous 16° temperature and at the other day-night combinations in which 16° was the day or night temperature, plants were stunted, with purplish stems and yellowish-green leaves, and disease development was decidedly inhibited. Under such conditions both nitrates and carbohydrates accumulated and protein synthesis was low (9, 13, 14, 15). At 20–24°, nitrate assimilation and translocation were faster, less carbohydrates accumulated, and plants grew vigorously. In similar temperature studies with angular leaf spot of cucumber, Van Gundy and Walker (12) found a combination of 16° day and 28° night most favorable for the disease. Thaung and Walker (11) found the same combination and a continuous 28° most favorable for bacterial blight of lima beans.

In plants supplied with various levels of N, P, and K, disease was markedly reduced by high N, low and high P, or high K. Bacterial spot of peach was enhanced by high N and retarded by high P (2). High N, low P, or low K favored bacterial blight of lima bean (11), and high N or low K favored angular leaf spot of cucumber (12). In tomato plants supplied with a nutrient solution high in osmotic value but otherwise balanced, plant growth was limited and disease development was markedly inhibited, paralleling the effects of high concentrations of any one of the elements N, P, and K. Magistad et al (8) reported that total salt concentration, expressed in atmospheres, was a greater factor in determining growth reduction

Table 5.—Development of bacterial spot on leaves of tomato plants grown in sand culture with high concentrations of N, P, and K, and with high osmotic values.

Nutrient levels (ppm)			Osmotic value (atm.)	Disease index[a]	Av. fresh wt. (g) of plants	
N	P	K			H[b]	D[c]
200	31	230	1.0	79	45	41
200	31	230	2.4	50	34	32
200	31	230	5.7	4	14	12
100	15.5	115[d]	1.0	79		
100	15.5	115	2.4	42	34	27
100	15.5	115	5.7	0	12	11
100	15.5	1600	2.4	54	28	24
1600	15.5	115	5.7	25	11	10
LSD	19:1			14		
	99:1			19		

[a] The values are averages of indices of 6 plants recorded 12 or 13 days after inoculation. 0 = no signs, 100 = 100 or more lesions per leaflet, extreme chlorosis and/or brown areas, conspicuous epinasty of leaflets and/or leaves.

[b] The values are averages of 2 healthy plants in each treatment.

[c] The values are averages of 6 diseased plants in each treatment.

[d] Basal nutrient.

than were specific ions. They also said that sodium did not appear to be an unduly toxic cation in sand cultures. Nightingale (10) reported that excess amounts of either nitrates or phosphates inhibited the uptake of each other, and that nitrate and potassium favored the intake of each other. It appears probable that the high osmotic values of the nutrient substrates and/or the presence of any one ion in very high concentration affect optimal absorption of the essential elements, and that the consequent imbalance in the host metabolism affects both plant growth and the pathogenicity of the bacterial spot organism.

The fact that older leaves are less susceptible to infection and that adverse conditions of temperature or nutrition retard the development of bacterial spot points to the importance of a change in the host metabolism induced by these conditions. In addition, the progress of disease through the 4 disease stages indicates a probable sequence of physiological changes in the host, with the eventual death of the affected part. A knowledge of such a change in host physiology may help in understanding how and why the disease develops in a susceptible host. It also holds the possibility of application of chemotherapy to economic advantage. Investigations of these aspects will be reported on later.

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GENETICS OF RESISTANCE IN CAPSICUM ANNUUM TO TWO VIRUS DISEASES

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SUMMARY

Resistance to tobacco etch virus in the P11 strain of *Capsicum annuum* was found to result from a single recessive gene, as did resistance to potato virus Y. The genetic factors that conferred resistance to the respective diseases in P11 were the same as those inherent in the S.C. 46252 strain of the same species. An apparent association of the genetic factor(s) conferring resistance to the 2 diseases was assumed to result from either close linkage of 2 distinct genes or the pleiotropic effects of a single recessive gene. Because recombinant genotypes could not be demonstrated, resistance to both virus diseases was considered to be the effect of the same single recessive pleiotropic gene in P11 and S.C. 46252. The designation ey^2 is proposed for this gene.

Introduction.—The discovery of multiple virus disease resistance to *Capsicum annuum* L. (3) provided plant breeders with new parental stocks. Before full use can be made of this material, however, an understanding must be attained of the basic inheritance and of the genetic relation of the factors conferring each of the individual resistances. Resistance to tobacco etch virus (TEV) in S.C. 46252, hereinafter referred to as SC, was found to result from a single recessive gene designated et^2 by Greenleaf (5). Immunity to potato virus Y (PVY) in both the P11 and SC peppers was attributed to another single recessive gene, y^2 (4). Resistance in *C. annuum* and *C. frutescens* L. to tobacco mosaic virus (TMV) was studied by Holmes (7, 8). Conclusions about the relation of the alleles involved, particularly that designated L, were based on information obtained with *C. frutescens*, but they apparently are also applicable to *C. annuum* (unpublished information).

Apparent similarities in degree and effectiveness of resistance in the 2 strains of *C. annuum*, P11 and SC, were noted by Cook and Anderson (3). The experiments herein reported were performed in an attempt to ascertain more exactly the relationship of the genetic factors conferring resistance to TEV and PVY in these 2 pepper strains.

Materials and methods.—The TEV and PVY strains used throughout these experiments were those previously designated TEV-E and PVY-N (3). The carborundum-gauze pad technique was used in all inocu-

Table 1.—Inheritance of resistance from P11 and SC to TEV-E.

Progeny	Seg. ratio	P11 No. plants Sus.	P11 No. plants Res.	P11 P values[a] T	P11 P values[a] P	P11 P values[a] I	SC No. plants Sus.	SC No. plants Res.	SC P values[a] T	SC P values[a] P	SC P values[a] I
F_1	1:0	72	3[b]				42	0			
F_1BC, sus.	1:0	254	0	.99	.99	.99	154	0	.99	.99	.99
F_1BC, res.	1:1	109	110	.7–.8	.95	.3–.5	67	45	.02–.05	.02–.05	
F_2, CW	3:1	101	24	.1–.2	.1–.2	.1–.2	79	30	.5–.7	.5–.7	.5–.7
F_2, FG	3:1	107	39	.3–.5	.5–.7	.2–.3					
F_2, IWB	3:1	128	39	.3–.5	.5–.7	.2–.3	104	41	.3–.5	.3–.5	.3–.5
F_2, YW	3:1	110	33	.5–.7	.5–.7	.3–.5	115	41	.1–.2	.7–.8	.05–.10
F_2, Total	3:1	446	135	.3–.5	.3–.5	.3–.5	298	112	.5–.7	.2–.3	.5–.7

[a] Respectively total, pooled, and interaction chi² values.
[b] F_1 test plants not reinoculated.

lations of test plants. Inoculum was prepared by triturating systemically infected leaves from recently infected tobacco plants (*Nicotiana tabacum* L. var. Turkish). Inocula were never diluted with water more than 1:25. Experiments with mixed inocula involved about equal amounts of tobacco leaf tissue infected with each virus for about the same length of time.

Test plants were inoculated in the 4–6-leaf stage first, and reinoculated after 21 days except where noted below. Plants were observed for at least 21 days after the second inoculation before virus recovery was attempted. In all experiments, a few plants of the variety Tabasco were included as a check on the infectivity of the inoculum as well as to verify identity of the virus. Plants of this variety were also used in virus recovery tests.

Inheritance of resistance from P11 and SC to TEV and PVY was determined by inoculation of F_1, backcross, and F_2 progenies. Both peppers were out-crossed to 4 varieties, each of which was susceptible to both virus diseases. These susceptible varieties were California Wonder (CW), Florida Giant (FG), Improved Worldbeater (IWB), and Yolo Wonder (YW).

Screening for cross-over genotypes was done by inoculation of F_2 progenies of P11 and SC out-crossed to the above 4 susceptible varieties. Test plants in these experiments were first inoculated in the 4–6-leaf stage. Within 14 days of inoculation the plants showing symptoms were discarded and the remaining plants were reinoculated with the same virus. After an additional 21 days, all plants exhibiting symptoms were, in turn, discarded and the remaining plants inoculated with the second virus. This procedure was tried with TEV-E inocula in the first 2 inoculations and a single PVY-N inoculation, and also in reverse order.

A progeny as herein defined constituted the plant population resulting from the seed complement of a single fruit, irrespective of whether the fruit was obtained from an out-cross, self-pollination, or back-cross.

All plants were grown in screened greenhouses to minimize the possibility of promiscuous cross-pollination and/or virus inoculation by insects.

Results.—From the data summarized in Table 1, it was concluded that resistance to TEV in the P11 and SC peppers was the expression of a single recessive gene pair. Furthermore, both peppers were homozygous for the respective factors. The portion of Table 1 pertaining to SC confirms the results obtained by Greenleaf (5).

Resistance in P11 and SC to PVY was also found to result from a single recessive gene pair (Table 2). Both peppers were homozygous in this respect. Only a single F_1BC resistant progeny was at hand for study of SC resistance, which may account for the significant P values.

To determine the identity of the P11 gene pair that effected resistance to TEV with respect to the corresponding gene pair from SC, 94 F_1 plants of P11 crossed by SC were inoculated with TEV-E. All of these plants proved resistant. Twenty plants were then

Table 2.—Inheritance of resistance from P11 and SC to PVY-N.

Progeny	Seg. ratio	P11 No. plants Sus.	P11 No. plants Res.	P11 P values[a] T	P11 P values[a] P	P11 P values[a] I	SC No. plants Sus.	SC No. plants Res.	SC P values[a] T	SC P values[a] P	SC P values[a] I
F_1	1:0	66	1[b]				48	0			
F_1BC, sus.	1:0	134	0	.99	.99	.99	183	0	.99	.99	
F_1BC, res.	1:1	128	138	.7–.8	.5–.7	.5–.7	30	48	.02–.05		
F_2, CW	3:1	106	42	.5–.7	.3–.5	.5–.7	198	62	.3–.5	.5–.7	.3–.5
F_2, FG	3:1	117	28	.2–.3	.1–.2	.3–.5					
F_2, IWB	3:1	154	54	.02–.05	.7–.8	.02–.05	124	56	.2–.3	.05–.10	.5–.7
F_2, YW	3:1	231	86	.3–.5	.3–.5	.3–.5	128	50	.3–.5	.3–.5	.3–.5
F_2, Total	3:1	608	210	.2–.3	.5–.7	.2–.3	450	168	.5–.7	.2–.3	.5–.7

[a] Respectively total, pooled, and interaction chi² values.
[b] F_1 test plants not reinoculated.

Table 3.—Results from inoculation of P11 and SC F_2 progenies with inocula of TEV-E and PVY-N mixed.

	No. plants		Seg.	P values[a]		
	Sus.	Res.	ratio	T	P	I
CW × P11	207	61	3:1	.5–.7	.3–.5	.3–.5
FG × P11	225	97	3:1	.1–.2	.02–.05	.5–.7
IWB × P11	229	68	3:1	.5–.7	.3–.5	.5–.7
YW × P11	189	81	3:1	.1–.2	.05–.10	.3–.5
Total, P11	850	307	3:1	.3–.5	.2–.3	.5–.7
CW × SC	229	71	3:1	.05–.10	.5–.7	.02–.05
FG × SC	218	93	3:1	.05–.10	.02–.05	.2–.3
IWB × SC	207	73	3:1	.5–.7	.5–.7	.3–.5
YW × SC	256	85	3:1	.90–.95	.99	.7–.8
Total, SC	910	322	3:1	.3–.5	.3–.5	.3–.5

[a]Respectively total, pooled, and interaction chi² values.

selected and allowed to set at least 2 selfed fruits each. The F_2 plants obtained from the seeds of a single fruit from each of these plants were inoculated with TEV-E. Symptoms were not observed in any of 576 such plants. Neither were symptoms noted nor virus recovered, in limited tests, from any of 719 F_2 plants derived from seeds of another fruit from each of the same 20 F_1 plants but inoculated with PVY-N. To relate the P11 gene that conferred resistance to PVY with the corresponding factor from SC, this inoculation procedure was reversed, PVY-N being used to inoculate 115 other F_1 plants. As with TEV-E, all plants were resistant, and 20 were selected for seed production as described above. None of the 556 F_2 plants, resulting from the seeds of a single fruit from each of the F_1 plants, was susceptible to infection with PVY-N. The 651 F_2 plants, secured from seeds of a second fruit from each of the F_1 plants, were all resistant to TEV-E. From these results, it was concluded that the recessive genes in the P11 and SC peppers that effected resistance to TEV were the same, as were the genes from the same 2 sources that conferred resistance to PVY. Furthermore, an association of the gene pairs governing resistance to TEV and PVY in both peppers became apparent.

Additional information in support of the above hypothesis was obtained with mixed inoculum. Instead of the 15:1 ratio of susceptible-resistant plants that would be expected in the F_2 if 2 gene pairs were inherited independently, the ratio obtained was about 3:1 (Table 3). Segregation according to this ratio could result from either of 2 genetic mechanisms: 1) close linkage of 2 distinct genes or 2) pleiotropic effects of a single gene. To determine which was in operation, attempts were made to demonstrate the occurrence of theoretical cross-over genotypes viz. $et^a et^a Y^a$– and/or et^{a+}–$y^a y^a$. Demonstration of either such genotype would have confirmed the presence of 2 distinct genetic factors involved in resistance to TEV and PVY, and would have made the theory of pleiotropism untenable in this instance. Attempts to demonstrate the occurrence of cross-over genotypes, however, were uniformly unsuccessful (Table 4). The resistance of the P11 and SC peppers to TEV and PVY was considered to result, then, from the pleiotropic effects of the same single recessive gene pair.

Discussion.—Though only 2 virus strains were used in these experiments, both TEV-E and PVY-N were considered representative of the respective viruses.

Mixed inoculum was used with full knowledge of the results obtained by Bawden and Kassanis (2) with a "severe etch virus" and PVY in tobacco. The effects of double infections of TEV-E and PVY-N in *C. annuum* plants susceptible to both viruses have more nearly resembled synergy, but apparent differences in varietal reaction have been noted (1).

For convenience in screening for recombinant genotypes, F_2 instead of back-cross progenies were used. Assuming the same percentage crossing-over in both types of progenies, only half as many plants remained to be inoculated with the second virus. Yield of cross-over genotypes distinguishable by the technique used would have been essentially the same from both types of progenies.

According to the analysis of pleiotropism and the possible methods of effect outlined by Grünberg (6), the example herein considered would properly be referred to as "spurious pleiotropism." This would imply at least 2 different effects emanating from a single gene and resulting from the same type of mechanism.

Until such time as the theory of spurious pleiotropism as an explanation for the virus disease resistance herein considered is disproved, it is proposed that the symbol ey^a be adopted to indicate this association of resistances.—Department of Plant Pathology, University of Florida, Gainesville.

Table 4.—Results of attempts to demonstrate recombinant genotype $et^a et^a Y^a$– and/or et^{a+}–$y^a y^a$.

	Number of plants of indicated genotypes determined from 2 inoculation procedures					
	TEV-E inoculations followed by PVY-N inoculation			PVY-N inoculations followed by TEV-E inoculation		
F_2 progenies	et^{a+}—	$et^a et^a Y^a$–	$et^a et^a y^a y^a$	—Y^a–	et^{a+}–$y^a y^a$	$et^a et^a y^a y^a$
CW × P11	141	0	51	75	0	17
FG × P11	287	0	99	135	0	40
IWB × P11	163	0	42	94	0	27
YW × P11	358	0	117	68	0	20
Total, P11	949	0	309	372	0	104
CW × SC	388	0	108	105	0	31
FG × SC	69	0	11			
IWB × SC	364	0	131	86	0	28
YW × SC	316	0	112	100	0	30
Total, SC	1137	0	362	291	0	89

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PRODUCTION OF PECTOLYTIC ENZYMES AND FUSARIC ACID BY FUSARIUM OXYSPORUM F. CONGLUTINANS IN RELATION TO CABBAGE YELLOWS

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SUMMARY

Fusarium oxysporum f. *conglutinans* and f. *lycopersici* were cultured on wheat bran, and on stem sections of cabbage and tomato sterilized with propylene oxide. Form *lycopersici* produced much more pectin depolymerase and pectin methyl esterase on each substrate than did f. *conglutinans*. Hydrolysis of pectate to galacturonic acid by f. *conglutinans* grown on wheat bran was demonstrated; f. *lycopersici* did not produce the monomer under the same conditions, but did so in small amount when spores from a culture grown on pectin medium were used as inoculum. The low DP activity of f. *conglutinans* and its degradation of pectate to galacturonic acid may be factors in the absence of wilt in cabbage yellows. Fusaric acid was produced by f. *conglutinans* in Richard's medium, but was not detected when the fungus was grown on cabbage stem sections. It was not detected in diseased host tissue, and was rapidly metabolized when introduced into cabbage cuttings. Since the acid could not be demonstrated in tissue of diseased cabbage by methods that have detected it in wilted cotton, this metabolite appears to be of little or no importance in the production of yellows symptoms.

Cabbage yellows, incited by *Fusarium oxysporum* Schlecht. f. *conglutinans* (Wr.) Snyd. & Hans., is similar to tomato wilt (*Fusarium oxysporum* f. *lycopersici* (Sacc.) Snyd. & Hans.) and cotton wilt (*Fusarium oxysporum* f. *vasinfectum* (Atk.) Snyd. & Hans.) in veinclearing and chlorosis of leaves and vascular browning, but differs in that affected leaves drop promptly, seldom wilting. The action of pectolytic enzymes has been shown to be important in tomato wilt (2, 11), and studies have been made of the role of fusaric acid in tomato wilt (1, 6), cotton wilt (5, 7, 8), and banana wilt (*Fusarium oxysporum* f. *cubense* (E. F. Sm.) Snyd. & Hans.) (9). Little is known of the role of these metabolites in cabbage yellows. Culture filtrates of the causal fungus have been shown to produce vascular browning in cabbage (12) and tomato (10), and small amounts of pectin methyl esterase (PME) and pectin-splitting enzymes have been demonstrated in such filtrates (12). Tomato cuttings developed no wilting after taking up spores of f. *conglutinans*, but wilted promptly after taking up spores of f. *lycopersici* (10). Production of fusaric acid by f. *conglutinans* has not been reported. This study was made to clarify the role of pectolytic enzymes and fusaric acid in the development of cabbage yellows.

Materials and methods.—Pectin methyl esterase (PME), determined by measuring the increase in acidity on hydrolysis (12), is expressed as microequivalents (μe) of methoxyl groups released from a 1% pectin solution (pH 5.5) by 1 ml of filtrate, when 1 part enzyme was incubated with 3 parts substrate and 0.3 parts of 0.5*M* acetate buffer (pH 5.5) for 3 hours at 30°C. Polygalacturonase (PG) activity, determined by iodometric titration (12), is expressed as μe of reducing groups liberated from 1% sodium polypectate (pH 5.5) by 1 ml of filtrate during incubation of 1 part enzyme with 2 parts substrate for 6 hours at 30°C. Depolymerase (DP) activity was determined by reduction of viscosity of a 1% sodium polypectate solution (pH 6.3). One part filtrate was used with 1 part substrate or, in case of wheat bran cultures, with 3 parts substrate. The activity is expressed as per cent reduction of viscosity during 2 hours' incubation at 30°C. The formation of galacturonic acid by PG was determined by paper chromatography in butanol–acetic-acid–water (4:1:5, v/v) after incubation of 1 part enzyme with 2 parts 1% sodium polypectate (pH 5.5) as substrate for periods of 1 and 2 days (2).

Production of fusaric acid.—Still cultures of f. *conglutinans* were grown on modified Richards' solution containing 5% sucrose (12). After incubation at

Table 1.—Activity of pectic enzymes in filtrates from cultures of *Fusarium oxysporum* f. *lycopersici* and f. *conglutinans* grown on different substrates.

Organism and substrate	DP activity[a]	PME activity[b]	PG activity[c]
f. *lycopersici*			
wheat bran	60.5	16.3	29.6
tomato stems	91.0	9.5	
cabbage stems	95.2	18.4	
f. *conglutinans*			
wheat bran	10.7	8.6	31.6
tomato stems	35.1	1.5	
cabbage stems	6.7	6.5	

[a] Depolymerase (DP) activity expressed as per cent reduction in viscosity of sodium polypectate within 2 hours.
[b] Pectin methyl esterase (PME) activity expressed as microequivalents of OCH_3 groups liberated from pectin solution within 3 hours.
[c] Polygalacturonase (PG) activity expressed as microequivalents of reducing groups liberated from sodium polypectate within 6 hours.

28°C for 7, 14, and 21 days, cultures were filtered and the filtrates assayed for fusaric acid by methods described by Zähner (13). An aqueous extract was also prepared from cultures of the organism grown for 10 and 14 days on cabbage stem sections sterilized with propylene oxide. Two g of charcoal were added to 100 ml of the extract; after shaking for 30 minutes the liquid was filtered off and the charcoal extracted successively with methanol ammonium hydroxide (100:10, v/v). This extract was concentrated *in vacuo* and subjected to descending paper chromatography in secondary-butanol–formic-acid–water (75:15:10, v/v). For detection of acidic spots the chromatograms were sprayed with bromocresol green. The presence of fusaric acid was also tested by bioassay, according to the method of Kalyanasundaram (4). Strips of the chromatograms or small cups filled with 0.2–0.4 ml of extract were placed on agar medium seeded with *Bacillus subtilis*, and growth inhibition at 37°C was evaluated. The identity of fusaric acid was confirmed by co-chromatography with pure fusaric acid and by its ultraviolet spectrum. Attempts failed to detect fusaric acid as its copper complex by the method of Lakshminarayanan and Subramanian (8). The quantitative assay of fusaric acid was carried out by spectrophotometric analysis according to a modification of the method of Zähner (13). The location of the acid on paper was marked under ultraviolet light. Areas containing the spots were cut out and eluted 5 hours at 70°C with 10 ml 95% ethanol. Together with 2 replicates of the unknown, 2 different known concentrations in duplicate and paper blanks were determined on each chromatogram. The extinction was measured at 268 mμ with a Beckman DU spectrophotometer. The maximum error was ±5%. Infected plants showing marked symptoms were extracted by grinding with sand and water in a mortar (5) or by boiling in 80% ethanol. The filtered and centrifuged extracts were concentrated *in vacuo* to 1/10 of the original fresh weight and subjected to paper chromatography and bioassay.

Experimental results.—*Activity of pectolytic enzymes.*—The data presented in Table 1 show that f. *conglutinans* as well as f. *lycopersici* produced pectolytic enzymes, though the two forms differed in activity of the enzymes. When grown on wheat bran, f. *conglutinans* as compared with f. *lycopersici* showed a considerably lower activity of PME and of DP as determined by reduction viscosity but the same PG activity as determined by liberation of reducing groups. Form *conglutinans* on cabbage stem sections showed more profuse mycelial growth than did f. *lycopersici* on tomato stems sections. Nevertheless the DP activity of the filtrate of tomato stems on which the tomato form had grown was nearly 15 times as great as the activity of filtrates from cabbage stems on which f. *conglutinans* had grown. With tomato stems as substrate the DP activity of f. *lycopersici* was nearly 3 times as great as that of f. *conglutinans*. The PME activity of the filtrates from substrates on which f. *lycopersici* was grown was several times as great as on the respective substrates on which f. *conglutinans* was grown.

It was not possible to measure PG activity reliably on stem section cultures of cabbage. In such cultures, oxidizing substances formed that interfered with the iodometric assay for PG, which is based on measuring the amount of reducing groups liberated by the enzyme action. These phenomena led to observations and studies concerned with oxidative enzymes in infected cabbage, which will be reported later (3). Culture filtrates from both forms grown for 5 days on wheat bran were tested for galacturonic acid liberated by the action of PG. When filtrates from f. *conglutinans* were used as the enzyme source, a very distinct spot corresponding to galacturonic acid was detected on the chromatograms at 24 and 48 hours. No galacturonic acid was formed by f. *lycopersici* when spores from a shake culture, with glucose as the carbon source, were used as inoculum for the wheat bran. When the inoculum was obtained from a shake culture with glucose plus 0.5% pectin, however, small amounts of galacturonic acid were liberated by the enzyme from the culture filtrate.

Fusaric acid production.—After growth of f. *conglutinans* for 7 days on Richards' solution, a trace of fusaric acid was detected in the concentrated filtrate by paper chromatography, and marked inhibition of *B. subtilis* was noted in the bioassay. Quantitative assays from paper chromatograms showed 182 mg of fusaric acid per L of filtrate at 14 days, and 206 mg/L at 21 days. When the fungus was grown on cabbage stem sections, the superficial mycelial growth reached a peak at 10 days and then tended to decline. When extracts of the substrate were concentrated and assayed at 10 and 14 days, no acid was detected either by paper chromatography or by bioassay.

Water and ethanol extracts were prepared from cabbage leaves with the characteristic symptoms of veinclearing and yellowing, and, after concentration, were tested for fusaric acid. The latter was not detected either by paper chromatography or by bioassay. In some cases very slight inhibition of bacterial growth occurred, but the bacteria rapidly grew over the

inhibition zone and even showed some evidence of stimulation. No sign of inhibition was observed when surface-sterilized stem or leaf tissue with evident yellows symptoms was placed on bacteria-seeded agar. The results suggested that fusaric acid was either not present or was formed in very low concentrations when the fungus was growing on stem sections or in the host plant. It is also possible that it was metabolized very rapidly and thus could not be recovered. To explore further into the latter possibility, experiments were carried out in which fusaric acid in concentrations corresponding to 200 mg/kg fresh cabbage tissue was added to macerated leaves and further preparation and concentration of the water extract was made immediately. Faint spots corresponding to fusaric acid could be detected on the chromatograms. With the bioassay method, inhibition of bacterial growth was very distinct. Cuttings obtained from young cabbage plants, 8–10 weeks old, were allowed to take up 100–200 mg of fusaric acid (pH 6.5) per kg fresh weight. Damage of veins appeared at 24 hours, and the symptoms were very pronounced at 48 hours, when slight yellowing of the leaves became evident and some were severely wilted. Neither with such cuttings nor with rooted plants that absorbed fusaric acid through a lower leaf petiole did the symptoms in any way resemble those of the naturally developing yellows disease. The cuttings treated with the toxin were extracted with water, and the concentrates were tested by chromatography and bioassay. No spots corresponding to fusaric acid could be detected on the chromatograms. Only very small zones of inhibition were obtained with *B. subtilis*.

Discussion.—In contrast to tomato wilt, cabbage yellows is characterized by veinclearing, chlorosis, and leaf drop, and wilting is rare. *Fusarium oxysporum* f. *conglutinans* produces much less DP and PME than does f. *lycopersici*. The latter organism shows especially high DP activity. The action of this enzyme gives rise to reducing groups too, but does not split the pectin molecule down to galacturonic acid, as does PG. Probably the most important difference between the 2 forms is in the DP and PG enzyme systems. In the present experiments no galacturonic acid was formed by f. *lycopersici*, except small amounts when spores from a pectin-containing medium were used as inoculum for the wheat bran culture. Waggoner and Dimond (11) demonstrated the formation of galacturonic acid when the fungus was grown on a pectin medium, but not on a substrate with glucose as a carbon source. This difference is not unexpected in view of the adaptive nature of pectolytic enzymes.

Form *conglutinans* shows very low depolymerase activity, but forms galacturonic acid by the action of PG even when the inoculum used is from a culture with glucose as the sole carbon source. This enzyme may be an exopolygalacturonase, which removes galacturonic acid from the end of the pectin chain. This would account for the equal amounts of reducing groups liberated by the enzymes of both forms. As pointed out by Gothoskar et al (2), however, the breakdown of the pectin molecule into large fragments by DP is most important as a cause of vascular plugging and wilt. The facts that the organism is capable of breaking pectin down to galacturonic acid and that DP activity is low may be factors responsible for the rarity of wilt in the symptom syndrome of cabbage yellows.

Evidence was clear that fusaric acid is produced by f. *conglutinans* when the fungus is grown on synthetic medium. Attempts failed, however, to demonstrate the presence of this toxin either in infected plants or in cultures of the organism on stem sections. Kalyanasundaram and Ram (5) used essentially the same techniques employed in this study. They demonstrated fusaric acid in concentrations of 17 mg/kg fresh weight of cotton leaves infected with f. *vasinfectum*. They also observed clear zones of bacterial growth inhibition when parts of infected leaves or stems were placed on bacteria-seeded agar. Their test organism for growth inhibition was an uncharacterized isolate from soil that showed a limit of sensitivity of 5 μg fusaric acid/ml, whereas the limit for the strain of *B. subtilis* used in this study was about 30 μg. Despite this difference the amount of toxin infiltrated into cabbage leaves was well within the range that could be readily detected. Though the concentrations (100–200 mg fusaric acid/kg fresh weight) taken up by cabbage cuttings were considerably higher than those found in cotton leaves, the inhibition of *B. subtilis* observed in extracts of such cuttings was very small. Only when extraction and analysis were carried out immediately after addition of the toxin could it be demonstrated in such extracts.

It is concluded that fusaric acid is very rapidly metabolized in cabbage plants. This applies also for stem section cultures, where fusaric acid might possibly be formed in low concentrations but is subjected to immediate decomposition and thus cannot be detected. Decomposition of fusaric acid is similarly rapid in tomato plants, where its presence as a vivotoxin could be established only by extremely sensitive methods, such as isotope dilution (1, 6). This rapid decomposition does not seem to be the case in cotton infected with f. *vasinfectum*, according to the results of Kalyanasundaram (5) and of Lakshminarayanan and Subramanian (7). When their results are compared with the findings for cabbage, it seems that fusaric acid certainly does not play an important role as a vivotoxin in cabbage yellows, as it apparently does in the case of cotton wilt. Further clarification of its role would require much more sensitive methods than were used by them and in this study.—Departments of Biochemistry and Plant Pathology, University of Wisconsin, Madison.

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OXIDATIVE ENZYMES IN CABBAGE INFECTED BY FUSARIUM OXYSPORUM F. CONGLUTINANS

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SUMMARY

Respiration and oxidative enzyme activity were studied in cabbage plants, resistant and susceptible to *Fusarium oxysporum* f. *conglutinans*, uninoculated and inoculated with that pathogen. Respiration was slightly increased in leaves of the susceptible variety 8 and 16 days after inoculation. The oxidation of ascorbic acid (oxidase activity) at 4 days was 2.5 times greater in inoculated resistant cuttings than in inoculated susceptible cuttings. At 8 days this activity had declined in resistant cuttings to that of healthy controls, but in susceptible cuttings it continued to rise. The activity was 41% and 113% higher in leaf extracts of inoculated susceptible plants than in comparable extracts of healthy plants at 8 and 16 days respectively. The total ascorbic acid content of susceptible inoculated plants at 14 days was about half that of healthy plants. Peroxidase activity at 8 days in inoculated susceptible cuttings was increased 132%. It is believed that the peroxidase was formed by the host, since culture filtrates showed a very large increase in peroxidase if the fungus had been grown on living stem sections, whereas no oxidase was detected if the fungus was grown on bran or autoclaved leaf extracts.

In recent years, special attention has been given to oxidative enzymes and their function in metabolic reactions in relation to disease development and resistance in plants. The literature on this problem was recently reviewed by Farkas and Kiraly (3) and by Uritani and Akazawa (21). Kiraly and Farkas (11) found an increase in ascorbic acid oxidase activity in homogenates of wheat infected with *Puccinia graminis* f. *tritici* Erikss. and E. Henn. Krupka (12) reported increased oxidation of ascorbic acid after the toxin victorin was taken up by oat varieties susceptible to *Helminthosporium victoriae* Meehan and Murphy. Cabbage infected with *Botrytis cinerea* Pers. was studied extensively by several Russians—Rubin and coworkers, cited by Farkas and Kiraly (3). In tissue around the infection site, they found a decrease of cytochrome oxidase and ascorbic acid oxidase activity in contrast to an increase in the activity of peroxidase.

Reduced ascorbic acid oxidase activity, together with an increase in phenoloxidase and peroxidase, was observed by Herzmann (9) in beans infected with *Colletotrichum lindemuthianum* (Sacc. and Magn.) Scribner. Umaerus (19) reported a positive correlation between peroxidase activity and field resistance of potato to *Phytophthora infestans* (Mont.) d By. Hassebrauk and Kaul (7) found that cytochrome oxidase and ascorbic acid oxidase activity was higher in uninfected wheat varieties resistant against brown rust, *Puccinia triticina* (*P. recondita* Rob. ex Desm. f. *tritici*), than in infected susceptible varieties. Resistant plants contained higher concentrations of ascorbic acid and phenolic compounds. In contrast, no consistent correlation between ascorbic acid content and resistance of wheat against stem rust was found by Pilgrim and Futrell (15).

Johnson and Schaal (10) reported that the ascorbic acid content of potato slices increased shortly after cutting and later decreased. They discussed the possibility that dehydroascorbic acid or other oxidation products may function in the resistance of plants to certain diseases.

The present work was initiated by observations made when *Fusarium oxysporum* Schlecht f. *conglutinans* (Wr.) Snyder and Hansen was grown on stem sections of cabbage so as to measure its pectolytic enzyme and fusaric acid production (8). Filtrates from such cultures revealed oxidizing substances that interfered with determination of the reducing groups. Very high activity of peroxidase was found in assays for oxidizing

enzymes, and differences were noted between the resistant and the susceptible variety. The activity of oxidative enzymes and oxygen uptake by respiration were therefore studied further in leaf tissue and cuttings of cabbage plants at different stages of disease. In addition, the concentration of ascorbic acid was determined in resistant plants and in susceptible plants uninoculated and inoculated with the pathogen.

Materials and methods.—Cabbage plants of the susceptible variety, Early Jersey Wakefield, and the resistant variety, Jersey Queen, were grown in a greenhouse maintained at 21±2°C. Stem sections from 8–10-week-old plants were surface-sterilized with propylene oxide. The fungus was grown for 3 days on such sections of living plant tissue, and culture filtrates were obtained by methods described elsewhere (8). Seedlings about 3 weeks old were inoculated by dipping the roots into a washed spore suspension from 3-day-old shake cultures of the organism on Czapek's medium and replanted in compost soil.

Cabbage plants were also grown in vermiculite in temperature-controlled growth chambers with 15-hour light periods (2000 ft-c) at 27±1°C and 9-hour dark periods at 20±1°C. Relative humidity was between 50 and 70%. Inoculations were carried out by cutting through the vermiculite close to 4–5-week-old plants and applying washed spore suspension with a pipette. The root-dipping technique could not be used, because of severe growth shock and wilting from transplanting under growth-chamber conditions.

Cuttings were obtained from 4–5-week-old plants grown in the growth chamber. The stems were cut under water and inoculated by being placed for 24 hours in small vials with a washed and filtered spore suspension containing about 100,000 spores per ml. The cuttings were covered with transparent plastic boxes to secure the high humidity necessary to prevent wilting. After 24 hours, they were transferred to half-strength Hyponex nutrient solution. The stems were cut under water, and the nutrient solution was changed daily.

Respiration measurements.—Leaves of healthy and infected plants were sampled between 9 and 10 A.M. when grown in the greenhouse, and 2 hours after the end of the light period when grown in the growth chamber. Following the method of Bonner and Wildmann (1), leaves were cut with a razor blade into pieces of about 2×2 mm and randomly mixed. Two-hundred-milligram portions in 4 replicates were transferred to Warburg vessels containing 2 ml 0.05*M* phosphate buffer (pH 4.5) with 0.15 ml 15% potassium hydroxide in the center well. Oxygen uptake was measured for 2 hours at 25°C. Aliquots of the leaf samples were used for dry-weight determinations, and O_2 uptake was calculated per 10 mg dry weight per 2 hours.

Enzymatic oxidation of ascorbic acid.—Three-gram leaf samples were ground at 4°C with sand and 6 ml 0.1*M* phosphate buffer (pH 6.5). The macerate was transferred with 3 ml buffer into centrifuge tubes for 15 minutes' centrifugation at 4000 G. An aliquot of the supernatant was diluted 1:1 with phosphate buffer. One ml of this extract was added to the Warburg vessel containing 0.8 ml 0.1*M* phosphate buffer (pH 6.5), with 0.5 ml ascorbic acid (24 mg/ml, brought to pH 6.0 with NaOH) in the side arm (11). After 15 minutes' equilibration, the substrate was tipped from the side arm, and oxygen uptake at 25°C was measured for 1 hour. Aliquots of the extracts were used for N determination with Nessler's method (20), the results being expressed as average O_2 uptake per 100 μg N during 1 hour in 3 or 4 replicates.

Determination of peroxidase.—A method described by Mudd et al (14) was applied. Aliquots of the extract used for the oxidation of ascorbic acid were further diluted 1:1 with 0.2*M* phosphate buffer (pH 6.0). One ml of this extract or 1 ml of the culture filtrate was used as the enzyme source. The reaction mixture in test tubes (150×15 mm) consisted of 2.6 ml water, 1 ml 0.1*M* phosphate buffer (pH 6.0), 0.4 ml 0.075% hydrogen peroxide, and 1 ml 0.01*M* pyrogallol. The pyrogallol was prepared fresh for every experiment. The enzyme was added, and the change in optical density in a Beckman model B spectrophotometer at 420 mμ was followed for 120 seconds at room temperature. Results were expressed as the change in optical density per 10 seconds per 100 μg N, calculated from the linear part of the curve during the first 70 seconds of the reaction. Maximum error was about ±10%, which was satisfactory for this purpose.

Determination of phenoloxidase.—The method used for determining phenoloxidase was that described by Goddard and Holden (6). Oxygen uptake of plant extracts buffered at pH 6 with 0.1*M* phosphate was measured manometrically when catechol, hydroquinone, and p-cresol, respectively, were used as substrate, with a trace of catechol as mediator of hydroquinone or p-cresol oxidation.

Determination of ascorbic acid.—The method of determining ascorbic acid was that described by Franke (4). Deionized water was used for all solutions. Three-gram samples of leaves in triplicate were quickly ground in a mortar with sand and 20 ml freshly prepared 5% metaphosphoric acid, diluted further with 20 ml 5% metaphosphoric acid, and made up to 100 ml with water. Aliquots of the filtered extracts were used for titration with 2,6-dichlorophenol-indophenol to determine the reduced ascorbic acid.

For determination of total ascorbic acid, the dehydroascorbic was first reduced by hydrogen sulfide. For this, the remaining filtrate was brought to about pH 4.5 with $CaCO_3$ and mercuriacetate and lead sodium acetate were added. Hydrogen sulfide was passed through this solution and precipitated for 20 minutes. After 18 hours in the dark, the precipitate was removed by filtration and oxygen-free N was passed through the clear solution for 20 minutes. Aliquots were used for titration with 2,6-dichlorophenol-indophenol. The results were corrected by adding 5% to the calculated values for total ascorbic acid, since after H_2S treatment, ascorbic acid is recovered only to 95%. The amount of dehydroascorbic acid was calculated

Table 1.—Oxygen uptake of leaf tissue from susceptible Early Jersey Wakefield plants at different days after inoculation with *Fusarium oxysporum* f. *conglutinans*.

Time after inoculation	mm^3 O_2 uptake/10 mg dry wt/hour		
	Healthy	Infected	% increase after infection
8 days	41.6±2.2[a]	47.3±2.1	14
12 days	38.7±2.2	45.0±0.6	16
16 days	42.7±0.9	49.0±1.3	15

[a] Standard deviation of sample mean.

from the difference between total ascorbic acid and reduced ascorbic acid.

Experimental results.—*Respiration of rooted plants.*—The effect of disease development on oxygen uptake of leaves of susceptible cabbage was tested repeatedly with plants grown under greenhouse conditions. The increase in respiration ranged from 18%, at 8 days after infection, to 39%, at 16 days. Variation in absolute O_2 uptake between different experiments was too large, however, to allow definite conclusions. Therefore further measurements were made with plants grown in growth chambers where temperature and light were controlled more accurately. At a light-period temperature of 27°C. about the optimum for development of cabbage yellows, some plants showed advanced stages of the disease in the form of severe yellowing and browning. For the analyses, plants were selected that had leaves that were chlorotic but not brown or severely damaged. As shown in Table 1, respiration increased slightly after infection under growth-chamber conditions. The difference is significant, though small compared to values obtained in other host-parasite complexes (2, 17).

Enzymatic oxidation of ascorbic acid.—Oxidation of ascorbic acid was first tested with leaf extracts from cabbage plants grown in the greenhouse. The increase in rate of oxidation ranged from 4%, at 8 days after inoculation of susceptible Early Jersey Wakefield plants, to 246%, at 16 days. Results were uniform with plants from the growth chamber. As shown in Table 2, extracts from infected plants showed an increase over extracts from healthy plants—41% 8 days after inoculation, and 113% 16 days after. Extracts from healthy leaves of rooted plants of the resistant variety, Jersey Queen, showed no difference from extracts from the susceptible variety, Early Jersey Wakefield.

When resistant cabbage plants were inoculated by root dipping and then replanted, the parasite did not invade the plant far enough to produce any symptoms or detectable metabolic alterations in the leaves. Therefore, cuttings of cabbage seedlings were inoculated by allowing spore suspensions to be taken up through the base of the cut stem. Cuttings of the susceptible variety showed typical vein clearing 7–8 days after inoculation, followed later by more pronounced chlorosis, browning, and wilting. These symptoms were not observed on cuttings of the resistant variety. They showed a diffuse type of chlorosis, starting at the veins, which tended to decrease later. The fungus could be reisolated from leaves of both susceptible and resistant cuttings 8 days after inoculation.

It thus became evident that the resistance reaction is not limited to roots or lower stems, but is also apparent in the leaves. This is in agreement with results obtained with tomato cuttings inoculated with *F. oxysporum* f. *lycopersici* (16). Therefore such leaves could be used for comparisons of metabolic differences between susceptible and resistant infected plants.

Leaf extracts from cuttings were tested 4 and 8 days after inoculation. Table 2 lists representative data from several consecutive experiments. Extracts from inoculated susceptible cuttings showed a 35% increase in oxidation of ascorbic acid over healthy controls 4 days after inoculation, and a 55% increase 8 days after. In similar comparisons of activity in inoculated and uninoculated cuttings from the resistant variety, the former showed 86% greater activity 4 days after inoculation and slightly less activity 8 days after inoculation.

To determine whether the oxidation of ascorbic acid in such plant extracts was due to an active ascorbic acid oxidase system, certain of the criteria outlined by Mapson (13) were used to show the presence of this enzyme. Sodium diethyldithiocarbamate (DDC) and ethylenediamine tetraacetic acid (EDTA) were added in various concentrations to extracts of infected plants. As shown in Table 3, DDC inhibited ascorbic acid oxidation when the concentration in the system was

Table 2.—Enzymatic oxidation of ascorbic acid by leaf extracts from rooted cabbage plants and cuttings of the resistant variety Jersey Queen (JQ) and the susceptible variety Early Jersey Wakefield (EJW), uninoculated and inoculated with *Fusarium oxysporum* f. *conglutinans*.

Source of extract	Days after inoculation	mm^3 O_2 uptake/100 μg nitrogen/hour		
		Healthy	Infected	% increase after infection
Rooted plants				
JQ		45.3±0.7[a]		
EJW	8	46.0±3.9	64.8±5.6	41
EJW	12	54.0±4.2	114.5±5.7	112
EJW	16	54.8±3.5	116.5±2.3	113
Cuttings				
JQ	4	53.7±1.2	100.0±2.6	86
JQ	8	59.6±0.6	54.4±2.3	9
EJW	4	47.0±0.65	63.3±2.2	35
EJW	8	56.1±1.5	86.7±1.5	55

[a] Standard deviation of sample mean.

Table 3.—Action of sodium diethyldithiocarbamate (DDC) and ethylenediamine tetraacetic acid (EDTA) on the enzymatic oxidation of ascorbic acid by leaf extracts from infected cabbage.

Substance and concentration added	O_2 uptake (mm^3/ml extract/hour)	Activity (% of control)
Control	112	100
DDC $10^{-2}M$	22	20
DDC $5\times10^{-3}M$	24	21
DDC $10^{-3}M$	76	68
EDTA $5\times10^{-3}M$	115	103
EDTA $10^{-3}M$	123	110
EDTA $10^{-4}M$	110	98

Table 4.—Activity of peroxidase in leaf extracts from rooted plants and cuttings of resistant Jersey Queen and susceptible Early Jersey Wakefield cabbage, uninoculated and inoculated with *Fusarium oxysporum* f. *conglutinans* and in filtrates from cultures of the fungus grown on different media.

Source of extract	Days after inoculation	Change in O.D. in 10 seconds[a]		
		Healthy	Infected	% increase after infection
Rooted plants				
JQ		0.054		
EJW	8	0.060	0.080	33
EJW	12	0.063	0.140	123
EJW	16	0.060	0.142	136
Cuttings				
JQ	4	0.045	0.054	20
JQ	8	0.044	0.053	20
EJW	4	0.036	0.045	25
EJW	8	0.038	0.050	32
Culture filtrates				
Stem sections, JQ	3	0.021	0.441	2200
Stem sections, EJW	3	0.013	0.308	2400
Wheat bran	3	0	0	
Autoclaved leaf extract	7	0	0	

[a] Change in optical density (O.D.) at 420 mμ per 10 seconds, calculated from linear part of curve between 40 and 70 seconds on base of 100 μg nitrogen in extract.

10^{-2}–$10^{-3}M$. EDTA did not influence O_2 uptake at any concentration tested from $5\times10^{-3}M$ to $10^{-4}M$. These results, together with the heat sensitivity of the oxidation, are evidence that ascorbic acid oxidase is active in extracts of cabbage leaves. Moreover, this enzyme has been demonstrated in cabbage by a number of workers (13).

Peroxidase.—Susceptible Early Jersey Wakefield plants grown under greenhouse conditions showed increases of peroxidase activity from 18% at 8 days to 94% at 16 days after inoculation. With susceptible plants grown in the growth chamber the increase ranged from 33% at 8 days to 136% at 16 days (Table 4). No significant differences were obtained with extracts from healthy leaves of the resistant and susceptible varieties.

Cuttings from both varieties were inoculated, and the activity of peroxidase was determined with leaf extracts at 4 and 8 days after inoculation. The increases at 8 days were 32% in the susceptible variety and 20% in the resistant variety (Table 4).

Culture filtrates obtained from 3-day-old cultures of f. *conglutinans* on stem sections of resistant and susceptible cabbage showed a very high activity of peroxidase—more than 2200% greater than that of the uninoculated tissue (Table 4). The increase after inoculation in culture filtrates from both varieties was about the same, though filtrates from the resistant variety showed a definitely higher absolute activity calculated on the basis of nitrogen content.

When the fungus was grown on wheat bran and on autoclaved leaf extracts, no peroxidase activity was detected in culture filtrates, though they showed a slightly darker color than did filtrates from the uninoculated controls. This indicated that in stem-section cultures the peroxidase was formed by the living plant tissue rather than by the fungus.

To characterize peroxidase activity in plant extracts further, several inhibition experiments were carried out (Table 5). The oxidation of pyrogallol by extracts from infected plants in the presence of H_2O_2 was completely inhibited by $10^{-3}M$ NaCN and considerably decreased at lower concentrations. In addition, considerable inhibition resulted when NaF and NH_2OH were present in the reaction mixture at concentrations between 10^{-2} and $10^{-4}M$. This indicated the presence of a metal enzyme, such as the iron-containing peroxidase.

Phenoloxidase.—Extracts of healthy and inoculated plants and culture filtrates were tested for the oxidation of phenols, but no measurable O_2 uptake was observed in manometric experiments either when the substrate was hydroquinone, catechol, or p-cresol or when trace amounts of catechol were added as a mediator for the hydroquinone or p-cresol oxidation. Filtrates from stem-section cultures showed a very small O_2 uptake when the substrate was hydroquinone with trace amounts of catechol. The activity, however, was not high enough for reliable measurements.

Determination of ascorbic acid.—Ascorbic acid concentration was determined in leaves of 6-week-old plants grown in the growth chamber. Healthy plants of the resistant and the susceptible variety were compared with inoculated susceptible plants 14 days after inoculation, at which time the latter showed vein

Table 5.—Action of inhibitors on the activity of peroxidase in leaf extracts from infected cabbage.

Substance and concentration added	Change in O.D./10 seconds[a]	Activity (% of control)
Control	0.155	100
NaCN $10^{-3}M$	0	0
NaCN $10^{-4}M$	0.018	11.6
NaCN $10^{-5}M$	0.104	67
NaF $10^{-2}M$	0.057	36.8
NaF $10^{-4}M$	0.090	58.1
NH_2OH $10^{-2}M$	0.054	35
NH_2OH $10^{-4}M$	0.083	53.5

[a] Change in optical density (O.D.) at 420 mμ per 10 seconds.

Table 6.—Concentration of ascorbic acid in uninoculated resistant Jersey Queen and susceptible Early Jersey Wakefield cabbage and in EJW cabbage 14 days after inoculation with *Fusarium oxysporum* f. *conglutinans*.

Source of sample	Ascorbic acid (mg/100g)	Total ascorbic acid (mg/100g)	Dehydro-ascorbic acid (mg/100g)	Dehydro-ascorbic acid (% of total ascorbic acid)
JQ, uninoculated	86.9±1.0[a]	104.2±0.6	17.3	16.6
EJW, uninoculated	72.2±4.5	81.9±1.2	9.7	11.9
EJW, Inoculated	37.0±2.4	45.3±2.2	8.3	18.3

[a] Standard deviation of sample mean.

clearing and slight chlorosis.

The difference between the resistant and susceptible varieties was marked (Table 6), the latter showing about 17% less ascorbic acid. Total ascorbic acid and reduced ascorbic acid were decreased considerably after infection, being about half as much as in leaves of infected plants. Differences also occurred in the amount of dehydroascorbic acid after infection. The proportion of dehydroascorbic acid in total ascorbic acid increased slightly. It is not clear, however, whether this amount of dehydroascorbic acid is present *in vivo* or whether it is formed during extraction. Under the conditions in which extraction was carried out, slight oxidation probably was not excluded entirely. This error is eliminated, however, by the determination of total ascorbic acid, the concentration of which differs as does ascorbic acid.

Discussion.—The results presented in this paper show that the respiration of cabbage leaves is slightly increased after infection with *F. oxysporum* f. *conglutinans*. The activity of ascorbic acid oxidase and peroxidase was considerably higher in extracts from such leaves than in extracts from healthy leaves. The infection also leads to a very pronounced decrease in the concentration of ascorbic acid.

Whether ascorbic acid oxidase, phenoloxidase, and peroxidase function as terminal oxidase in the respiration of plants is still open to discussion (13, 21). The ability of plant extracts to oxidize phenols or ascorbic acid does not mean that the responsible enzymes are also active *in vivo*. This can be determined only by very careful studies with specific inhibitors or by testing respiration under different O_2 tension (21).

Our results therefore do not allow a final decision on which of these oxidative enzymes is activated *in vivo*. The decrease in ascorbic acid and the slightly enhanced respiration after infection, however, justify the conclusion that oxidative enzymes are activated *in vivo*. Ascorbic acid can be oxidized directly, by ascorbic acid oxidase, or indirectly, by the products of peroxidase action, both of which enzymes showed increased activity in extracts from infected plants.

The different ways in which resistant and susceptible cuttings react in metabolic activity to inoculation are probably most important in respect to explanation of the resistance reaction. Cuttings of the susceptible variety responded slowly, but enzyme activity continued to rise. Resistant cuttings responded rapidly, with a considerably greater increase in the activity of ascorbic acid oxidase than that in susceptible cuttings, but the activity declined later.

The infection of rooted plants is also characterized by a higher peroxidase activity, though the results with cuttings showed no clear-cut correlations between the resistant and susceptible varieties. The higher activity of peroxidase in culture filtrates from stem sections of the resistant variety, however, might suggest similar relations.

More rapid and intensive metabolic response to infection in the form of a hypersensitive reaction has been reported for resistant as compared with susceptible varieties in several host-parasite combinations. Literature on the possible mechanisms by which the resistant host may counteract the attack of the parasite is discussed in detail by Uritani and Akazawa (21) and Farkas and Kiraly (3). In some cases the accumulation of oxidation products and their inhibitory action on growth of the parasite could be demonstrated. In other cases the resistance might be connected with the oxidation of toxins secreted by the parasite. It seems evident, however, that similar processes go on in both resistant and susceptible plants; the main difference is in the rate and intensity of response to parasitic attack (5). This determines whether the balance between both systems shifts in favor of the parasite or the host, the latter shift resulting in elimination or effective retardation of the parasite.

Metabolic reactions in the ascorbic acid oxidase system seem especially worthy of further investigation. Our studies demonstrated no accumulation of dehydroascorbic acid, which Johnson and Schaal (10) thought might play a role in the resistance reaction. In cabbage, dehydroascorbic acid seems to be rapidly further metabolized via 2,3-diketogluconic acid or other pathways. In preliminary experiments concerning this question, we could demonstrate no differences in the enzymatic reduction of dehydroascorbic acid by glutathione. For future work, however, much more attention should be given to these more specific reducing systems that might influence the extent of oxidation. If oxidizing enzymes and their products play a role in the resistance reaction, the most crucial point is how their activation may be triggered or possibly counteracted. The resistant variety Jersey Queen contained higher concentrations of ascorbic acid, as also found by Smith and Walker (18), but we do not believe this is a determining factor for resistance. The enzymatic reactions connected with the oxidation of ascorbic acid and the reduction of dehydroascorbic acid are likely to be more important.—Departments of Biochemistry and Plant Pathology, University of Wisconsin, Madison.

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EVOLUTION OF SEXUALITY AND PATHOGENICITY. I. INTERSPECIFIC CROSSES IN THE GENUS HELMINTHOSPORIUM

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SUMMARY

Of 2366 interspecific crosses of related species of *Helminthosporium*, 50 succeeded, representing 13 different interspecific crosses. Perfect stages of the species used were in *Cochliobolus* or unknown. Viable ascospores were obtained from 9 of the 13 crosses, but the numbers of asci and ascospores produced varied markedly. Since all fertile crosses produced ascospores or asci typical of *Cochliobolus*, the species of *Helminthosporium* with no known perfect stage probably have *Cochliobolus* perfect stages. Preliminary backcross studies demonstrated that some ascospore progeny were fertile with one or the other of the parental strains, whereas others did not mate with either parent. Limited studies with progeny of *H. maydis* × *H. oryzae* have shown that some were pathogenic to either rice or corn, and others were pathogenic to both.

Although species of fungi are classified largely on the basis of morphological characters, the almost universal interspecific sterility among even related species probably results, at least in some instances, from genetic differences at one or more loci controlling the sexual mechanisms. A study of differences and similarities in the genetic systems controlling the sexual mechanisms of related species should provide important information on the pattern and direction of evolutionary changes.

In the Ascomycetes, the genus *Cochliobolus* includes species with coiled ascospores and *Helminthosporium* imperfect stages. Limited information on the basic pattern of sexuality has been reported for 4 of the heterothallic species. In *Cochliobolus heterostrophus* (Drechs.) Drechs., *C. carbonum* Nelson, *C. sativus* (Ito & Kurib.) Drechs ex Dastur, and *C. nodulosus* Luttrell, mating type or compatibility appears to be conditioned by a single major gene locus (1, 3, 8, 9). Variability in degree of compatibility among isolates has been demonstrated in *C. heterostrophus* (4) and observed in the other species, indicating that additional genes influence mating capacity. The presence of gene blocks at perithecial initiation, plasmogamy, and karogamy in *C. heterostrophus* further illustrates the complexity of the sexual mechanism in this group (5, 6, 7). In spite of this complexity, an apparent similarity in the basic patterns of sexual reproduction, coupled with a distinct similarity in the conidial morphology of the *Helminthosporium* conidial stages, suggested that fertile interspecific crosses might be possible. This paper reports the progress of these studies.

Materials and methods.—The following species of *Helminthosporium* were used: *H. maydis*, *H. carbonum*, *H. oryzae*, *H. sativum*, *H. setariae*, *H. victoriae*, *H. sorghicola*, and *H. sacchari*. Whenever possible, different isolates of each species were obtained from several geographic areas in an effort to obtain a broad genetic base for each species. In addition, 5 isolates of *Helminthosporium*, 2 each from rice and oats and 1 from Bermuda grass, were used. These isolates were considered to be sufficiently different morphologically from described species reported pathogenic to those hosts.

The pathogenicity of each of the isolates on its specific host was verified in inoculation studies. Though not all isolates were tested on all hosts, the cross-inoculations attempted were unsuccessful, which concurs with present concepts of the relatively rigid host specificity of this series of *Helminthosporium* species pathogenic to gramineous hosts.

Interspecific matings were made by placing small

Table 1.—Successful interspecific crosses of species of *Helminthosporium*.

Cross	Farthest stage of development	No. of crosses Attempted	No. of crosses Successful
H. maydis × *H. oryzae*	ascospores	627	2
H. maydis × *H. sorghicola*	ascospores	57	2
H. maydis × *H. carbonum*	ascospores	630	2
H. maydis × *H. sacchari*	asci	57	2
H. maydis × *H. sp.* 1 (rice)	ascospores	57	1
H. maydis × *H. sp.* 2 (rice)	ascospores	57	1
H. carbonum × *H. sativum*	asci	240	3
H. carbonum × *H. victoriae*	ascospores	600	31
H. setariae × *H. sativum*	asci	8	1
H. carbonum × *H. sp.* 5 (Bermuda)	asci	30	2
H. sp. 1 (rice) × *H. sp.* 5 (Bermuda)	ascospores	1	1
H. sp. 2 (rice) × *H. sp.* 5 (Bermuda)	ascospores	1	1
H. sp. 1 (rice) × *H. sp.* 2 (rice)	ascospores	1	1
Totals		2366	50

pieces of mycelium on opposite sides of a section of sterile corn leaf in the center of a Petri plate containing Sach's nutrient agar. Frequently, sterile barley grains were placed adjacent to the section of corn leaf. The sections of corn leaf and the barley grains were sterilized by autoclaving for 1 hour. All matings were incubated continuously at 24°C.

Results and discussion.—To date, 13 fertile interspecific crosses have been obtained (Table 1). Viable ascospore progeny have been isolated from 9 of the crosses, but the other 4 crosses have failed to develop beyond the production of mature asci. It is not known whether this failure represents inadequate techniques or a genetic block in reproduction.

Perithecial production was abundant in crosses of *H. carbonum* × *H. victoriae*, *H. maydis* × *H. oryzae*, *H. maydis* × *H. sorghicola*, and *H. maydis* × *H. sp.* 1 (rice), and scant in the other crosses. Numerous asci were produced in all crosses except *H. setariae* × *H. sativum* and *H. carbonum* × *H. sativum*. Ascospore production was abundant in *H. carbonum* × *H. victoriae*, and scant in the other crosses, and most of these did not germinate.

Preliminary studies on the compatibility reaction of ascospore isolates demonstrated that some were cross-fertile with one or the other of the parental isolates, whereas others did not mate with either parent. It is interesting to speculate whether the isolates that failed to cross with either parent represent 1 or more distinct species. From the standpoint of disease potential, this could be evaluated by inoculating a wide variety of gramineous species. If different hosts were susceptible, it would then be necessary to determine whether other species of *Helminthosporium* are known to attack those hosts. If such were the case, critical comparisons of morphology and attempted crosses should determine whether they are similar or different. All 13 interspecific crosses produced perithecia and asci typical of the genus *Cochliobolus*, and the ascospores produced in the 9 crosses were also representative of the genus. The results suggest that the species of *Helminthosporium* used in these studies that have no known perfect stage probably have perfect stages in *Cochliobolus*. The perfect stage of *H. victoriae* was obtained indirectly through crosses of *H. carbonum* × *H. victoriae*. It was observed that certain wild-type isolates of *H. victoriae* were compatible with 1 mating type of *C. carbonum*, and others were cross-fertile with the other mating type. The perfect stage of *H. victoriae*, a species of *Cochliobolus*, was then obtained simply by pairing the strains of opposite mating type.

There can be little doubt that success in genetic studies such as these depends primarily on using large numbers of isolates from diverse geographic areas. Nine of the 13 interspecific crosses involved *H. Maydis* or *H. carbonum*, and 87 of the 134 isolates used were of these 2 species. It is likely that as additional isolates of other species are obtained, other fertile interspecific crosses can be obtained. The fact that interspecific crosses are possible with this group of species indicates a similarity in compatibility mechanisms. It demonstrates, however, that compatibility *per se* is governed not by a single gene but perhaps by a single complex locus or a series of separate loci. To illustrate this point, 1 isolate of *H. oryzae* was cross-fertile with only 2 of 31 *A* isolates of *H. maydis*. Although it has been shown that a mutant "i" gene blocks the formation of protoperithecia in *H. maydis* (7), none of the *A* conidial isolates of that species carry the inhibitor gene in its recessive state, thus eliminating the possibility of this type of gene block in sexual reproduction. The compatibility of certain isolates of one species with certain isolates of another species further illustrates that all strains of one mating type are not the same genetically at all loci directly or indirectly conditioning compatibility.

A study of ascospore progeny obtained from interspecific crosses should provide valuable information on the evolution of pathogenicity in the series of *Helminthosporium* species attacking gramineous hosts. Preliminary studies with ascospore progeny from *H. maydis* × *H. oryzae* have shown that some of the progeny were pathogenic to either rice or corn, whereas others produced disease symptoms on both. Progeny pathogenic to both rice and corn could have been produced as a result of crossing over, which may have recombined genes for pathogenicity or eliminated suppressor genes. Furthermore, it is possible that these species of *Helminthosporium*, although relatively host-specific, may have genes in common for pathogenicity, particularly in view of the fact that pathogenicity in this group appears to be controlled quantitatively.—North Carolina State College, Raleigh.

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PATHOGENICITY AND STABILITY OF STRAINS OF CORYNEBACTERIUM INSIDIOSUM

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SUMMARY

Alfalfa clones selected from variety Du Puits, used commercially in western Europe and the U. S., were tested for susceptibility to bacterial wilt with mass isolates and selected strains of *Corynebacterium insidiosum*. Disease reaction indicated differential pathogenesis among strains of this organism. Seven of the clones selected were readily self-fertile and produced S_1 seed. These S_1 seedling populations were subsequently tested against a differentiating strain of the wilt organism. One of the S_1 populations was as uniformly susceptible as its parent clone, whereas another exhibited a large increase in susceptibility. No change was evident in the susceptibility of the other 5 seedling populations when they were compared with their respective parent clones. Some clones differed appreciably in agronomic characteristics and could be readily recognized on that basis, but they did not differ in susceptibility to bacterial wilt isolates used in these tests. Lack of pronounced cultural stability was evident in most isolates of *C. insidiosum* included in this study. When isolates were maintained 448 days in continuous culture at optimal and suboptimal temperatures, variants were detected in nearly all cultures.

Bacterial wilt of alfalfa (*Medicago sativa* L. and *M. falcata* L.) and its causal organism (*Corynebacterium insidiosum* (McCulloch) Jensen) have been well known in North America for 35 years. Since recognition of the disease (6) and identification and description of the causal organism (8, 10), bacterial wilt has been known almost entirely as a disease of perennial alfalfas, with few reports of the organism attacking other hosts (8, 18). The disease has been prevalent in many alfalfa-growing areas of the United States and Canada, and was recently reported from Italy (15). There reportedly are indications that the disease has been present in Chile for some time (4). Soon after its discovery, *C. insidiosum* was identified in diseased alfalfa roots from Turkestan (7). The organism was identified from grass sods in Australia (5), but it has not been known as an agent of alfalfa disease in that part of the world. Alfalfa plants exhibiting symptoms of bacterial wilt have been reported from England (11), but the associated bacteria were not found to be *C. insidiosum*, and the disease as such has apparently not been recorded from western Europe.

Conditions influencing the general development of alfalfa bacterial wilt have been studied (9, 13, 17), and histological work has afforded understanding of the progress of this disease in affected plants and probable modes of spread (1, 12, 14). The disease has a long, established record of economic importance in alfalfa culture in North America, and resistant varieties have afforded the most practical measure of control recognized thus far. The variation in wilt susceptibility of commercial varieties grown under different conditions of culture has raised the question of variability in the causal organism. This study was made to determine whether pronounced variability occurred in the pathogenicity of *C. insidiosum*; develop clonal or seedling populations for use in evaluating isolates of the organism; and obtain some measure of its cultural stability.

Materials and methods.—The studies reported herein were conducted in the laboratory and greenhouse. Plants to be tested were grown in sterilized pots and soil at about 24°C[1] with nitrogen fertilizer and artificial illumination supplied to maintain vigorous growth. The plants were cut at an early-bud stage to a height of 4 in., and regrowth was examined for symptom development.

Inoculations were accomplished by immersing washed root systems of plants in standardized suspensions of inocula and repotting before incubation. The length of this inoculation or immersion period was 12 hours, except where it is otherwise indicated. Isolates used for preparation of inocula were incubated 4–7 days at 21°C on beef-lactose agar (BLA) in plate cultures, or in nutrient lactose-broth (LB) shake cultures. Cells were then harvested and washed by centrifugation, resuspended in sterile distilled water, and standardized at approximately 10^6 cells per cc of suspension.

In some tests plants were classified on the basis of both top and root symptoms, but top symptoms were found more reliable for the plants used in this study. The classifications (Fig. 1) were based on a scale of 0 (no plant symptoms) to 3 (dead plant), and control plants were used to establish the general performance index for the particular seedling or clonal population being tested. Reisolations were made from both tops and roots to determine the presence or absence of the

[1]All temperatures are given in degrees Centigrade.

pathogen in inoculated and uninoculated plants.

Clonal propagates obviated the problem of variable susceptibility attributable to genetic differences inherent in seedling populations of common alfalfa varieties. These clones, previously described (2), were selected from a seedling population of Du Puits alfalfa. A mass isolate of *C. insidiosum* was used in the screening procedure and 7 clones were subsequently selected, 4 being used most commonly in tests described below.

Results.—*Variable pathogenicity.*—Clones DP_1 and DP_2 were used in tests designed to determine the relative virulence of 2 isolates of *C. insidiosum*. These isolates (34 and 34-11) could be readily distinguished in BLA culture and were maintained through single-colony transfer for 30 days before the preparation of inocula. Inoculated plants of the 2 clones in lots of 35 each, replicated 3 times, were incubated for 62 days. The results are summarized as follows: Clone $DP_1 \times$ isolate 34 = 1.4 mean index; $DP_1 \times 34\text{-}11 = 1.3$; $DP_2 \times 34\text{-}11 = 1.4$; and $DP_2 \times 34 = 0.5$ (LSD = 0.5**). Isolate 34 was less virulent in inoculated plants of clone DP_2, whereas the other 3 combinations resulted in about equal levels of pathogenesis.

Isolate 34 was similarly retested on 2 moderately susceptible clones (DP_3 and DP_4). With 3 replications of 100 plants per clone the mean indexed response was 0.81 for DP_3 and 0.88 for DP_4. The foregoing 2 clones were readily distinguishable on the basis of gross morphology, but no significant difference in susceptibility was indicated under the conditions of these tests.

Susceptibility of S_1 populations.—Seven Du Puits clones, including the 4 previously described, were found to have some susceptibility to bacterial wilt as well as being self-fertile. Selfed seedling-lines were produced from these clones and tested for uniformity of response to isolate 34 of the wilt organism. Table 1 summarizes the results of one such test, with standardized inoculum, root inoculation, and 80 days' incubation in the greenhouse. Two S_1 populations (SDP_1 and SDP_2) were distinctly susceptible to isolate 34; plants of SDP_1 behaved much like the parent clone, whereas plants of SDP_2 were more susceptible than the parent clone. No change in susceptibility relative to the respective parent clones was indicated by the other five S_1 populations.

Length of inoculation and incubation periods.—Varying the duration of inoculation (1/60, ½, 1, 4, and 18 hr.) did not consistently affect disease development or incidence in susceptible clonal plants. Immersion for 1/60, ½, or 1 hour was most frequently effective.

The incubation time required for inoculated plants to exhibit symptoms varied somewhat with the test clones and isolates used. Symptoms were seldom con-

Fig. 1.—Two plants from a susceptible clone of alfalfa (Du Puits) showing symptoms (index of plant on right, 2) and no symptoms (index of plant on left, 0) of bacterial wilt.

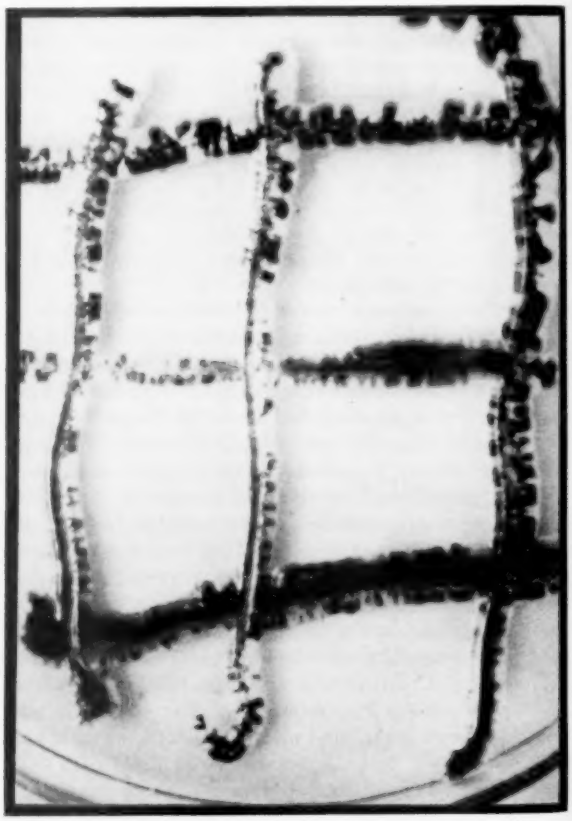

Fig. 2.—Plate culture of *Corynebacterium insidiosum* showing nonfluidic growth of deeply pigmented and nonpigmented variants.

Table 1.—Disease index of seven S_1 populations derived from selected clones of Du Puits alfalfa[a].

Seedling line no.	Mean disease index[b]
SDP_1	1.56
SDP_2	1.72
SDP_3	1.11
SDP_4	0.50
SDP_5	0.72
SDP_6	0.11
SDP_7	0.22

[a]Eighteen plants per seedling line.
[b]Indexed as 0 (no plant symptom) to 3 (dead plant). LSD = 0.43**.

spicuous until 36 days after inoculation, and expression generally reached maximum after 60 days.

Corynebacterium insidiosum in culture.—Development of blue, granular, extra-cellular deposits in cultures of *C. insidiosum* (8) has long been a distinctive diagnostic characteristic of this organism. It has been recognized (16) as variable even under favorable conditions of culture. Granule production is usually pronounced on BLA, but in this study the rate of development was variable among isolates, even at constant optimal temperature. Granules were not detected macroscopically in cultures less than 5 days old when incubated at 21°. When mass isolates from diseased tissue were used, 17 days were required for 80% of the isolates to become granule-positive. Some mass isolates did not become granule-positive until they were incubated 26 days. It is known that factors other than those inherent in the isolates may also influence granule production (16).

To determine the degree of stability in culture, three fluidic[2] and 3 nonfluidic isolates were each propagated through 5 single-colony generations. All 6 isolates replicated 5 times were plate-cultured on BLA at 21° in the absence of light. These cultures were examined and subtransferred on an average of once each 14 days for 448 consecutive days. Subtransfers were made by the dilution streak method, and obvious variants (Fig. 2) were thus removed. Concurrently, replicates of all 6 isolates were maintained on sealed BLA slants at 2–4°. Subcultures of the bacteria incubated at 2–4° were prepared after 132 and 420 days in storage and compared with the systematically transferred isolates incubated at 21°. The summarized results of these comparisons were as follows: (a) no fluidic variants were found in cultures of nonfluidic isolates; (b) most of the nonpigmented[3] variants occurred in cultures of nonfluidic isolates; and (c) nonfluidic variants were detected in nearly all cultures derived from both fluidic and nonfluidic isolates. In addition, 1 subculture each of the fluidic and nonfluidic type, concurrently maintained through selective transfer, was checked for pathogenicity at the beginning of the test period and after 365 days of culture at 21°. No change in virulence was detected in these 2 isolates.

Conclusion.—No isolate was invariably most virulent on all test clones of alfalfa. Two isolates were equally pathogenic on plants of one clone, but differed in pathogenicity on plants of another. These 2 clones were similar in general agronomic characteristics and over-all gross morphology. Two other clones, which differed phenotypically, were also tested against the more virulent of the 2 isolates described. Both of these clones were moderately susceptible. Thus, within the relatively narrow genetic restrictions of an alfalfa variety, isolates of *C. insidiosum* were found to exhibit a differential type of pathogenesis.

The S_1 seedling populations derived from 7 of the test clones were not invariably more susceptible than their respective parent clones. In 1 of the 7 populations the susceptibility was greater than that noted for the parent clone inoculated with the same isolate. Although the response of the plants within each of the populations was relatively uniform, selfing, at least in the first generation, did not generally result in plants with a level of susceptibility greater than the level previously established for the isolate used.

The apparent lack of stability of isolates of *C. insidiosum* under prolonged culture was greater than anticipated. Even when cultures were kept at low temperature, variants were detected. The occurrence of nonfluidic, deeply pigmented variants in a fluidic population has been observed to be of general occurrence. Occurrence of nonpigmented forms (3) in both fluidic and nonfluidic cultures requires further investigation. Since the nonpigmented forms grew readily in culture with other types, prolonged culture was not required for macroscopic detection. It is assumed that these types arise as independent variants and are relatively stable (16).—Crops Research Division, ARS, U.S.D.A., Beltsville, Maryland.

[2]The terms "fluidic" and "nonfluidic" are used herein solely to indicate the relative fluid or nonfluid characteristic of bacterial colonies growing on solid substrata.

[3]Nonpigmented is herein used only in the sense that such growth does not contain the blue, granular deposits characteristic of *C. insidiosum*. The nonpigmented growth may be white, faintly pink, or variously yellow to orange in color.

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HEAT INACTIVATION OF STONE FRUIT RINGSPOT VIRUS

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SUMMARY

Stone fruit ringspot virus was consistently inactivated by treatment at 100°F for 2 weeks in cherry and 3 weeks in peach, with simultaneous elimination of sour cherry yellows, prune dwarf, peach necrotic leafspot, Muir dwarf, and peach stunt diseases. Evidence was obtained that these diseases are caused by the ringspot virus. The appearance of flushes of symptoms of peach necrotic leafspot during the summer months is probably the result of temporary heat inactivation or immobility of ringspot virus in growing shoot tips followed by invasion of healthy tissue produced during the inactivation period.

Heat treatments of growing trees to cure several virus diseases of peach were reported by Kunkel in 1936 (4). He also reported heat inactivation of a virus of herbaceous plants in 1941 (5). A number of other investigators have attempted heat inactivation of plant viruses in growing plants, some with considerable success (2, 3, 8, 9, 10). This paper reports successful heat inactivation in several Prunus hosts of stone fruit ringspot virus along with elimination of sour cherry yellows, prune dwarf, Muir dwarf, peach stunt, and peach necrotic leafspot. Evidence indicates that these diseases are caused by the ringspot virus.

Materials and methods.—The trees treated were grown in clay pots or metal cans in a lath house or greenhouse. The containers varied from 6-in. pots and 1-gal. cans to 10-in. pots and 3-gal. cans. Sweet and sour cherry buds were propagated on mahaleb rootstock, and budlings were grown at least 3 months before treatment. In most cases the terminal buds of the plants had set. The peach trees, either Lovell seedlings or varieties budded on Lovell seedlings, had been established in the containers for at least one growing season. In some tests dormant trees were treated before bud break. In 1956 when these tests were begun, there were no provisions for control of light or humidity. In later tests, about 550 ft-c of light were available at plant height and relative humidity was maintained at 65–75% at 100°F. In these later tests constant temperature was maintained during the summer by a refrigeration system working against strip heaters. In most experiments the temperature was 100±2°F. Plants undergoing treatment were watered as necessary, from containers in the treatment room. In one experiment, by arranging partial stratification of the air, the soil was maintained at 85°F while air temperature at the plant tops was 100°F.

Before treatment, all plants were systemically infected with ringspot as indicated by local necrotic reaction in Shiro-fugen flowering cherry (7).

After the various treatment periods, portions of branches were removed as samples from treated trees. Buds from the samples were inserted into suitable rootstocks, and also into Shiro-fugen and in some cases other indicator plants for indexing. In other cases entire trees were removed from the heated room at desired intervals and placed in a greenhouse.

Results.—On August 6, 1956, 4 trees of Stockton Morello cherry on mahaleb rootstock were placed in a room maintained at 100°F in the vicinity of the branches. On August 23, 1 tree was removed to the greenhouse, and samples were taken as bark chips and buds and placed in Shiro-fugen. These samples indexed negative for ringspot. Shoots produced from latent buds in the treated Stockton Morello tree were removed 3 weeks after treatment and placed in a sand medium under mist to induce rooting. No roots formed but some lateral buds matured sufficiently to be propagated on mahaleb rootstock in the nursery. Internodal tissue from these shoots, taken at the same time as the buds, was again indexed on Shiro-fugen and was negative for ringspot. Four trees of Stockton Morello, obtained from the buds, have consistently tested negative for ringspot for 3 years.

The 3 remaining plants of this treatment were left in the heated room for 34 days. The one plant that survived was negative for ringspot until the spring of 1957, when shock symptoms appeared on early-season growth.

This test demonstrated that the ringspot virus could be inactivated in portions of the trees and that ringspot-free trees could be obtained from an infected source by this method.

Table 1.—Heat inactivation of the ringspot and peach necrotic leafspot complex in peach.

Variety	Diseases present	2 weeks		3 weeks		4 weeks	
		Pos.[a]	Neg.	Pos.	Neg.	Pos.	Neg.
Paloro	ringspot and peach necrotic leafspot	not done		2	0	2	6
Double Delight	ringspot and peach necrotic leafspot	not done		3	2	0	27
Muir	peach necrotic leafspot, Muir dwarf, and ringspot	5	0	5	4	0	9
J. H. Hale	ringspot and peach necrotic leafspot	14	0	0	6	2	24
Peak	ringspot, stunt (P.D.), and peach necrotic leafspot	not done		1	4	0	14

[a] Local necrotic reaction on Shiro-fugen flowering cherry. Numbers shown in these columns refer to numbers of buds or bark chips indexed.

A second experiment, started on February 7, 1957, involved Stockton Morello, Mapes sweet cherry, and Lovell peach seedlings known to be carrying ringspot virus from a culture also capable of causing sour cherry yellows, prune dwarf, and peach stunt. Soil temperature was 85°F. and air temperature in the vicinity of the branches was 100°F. Samples were removed after 17, 24, and 31 days. Some buds or bark chips were placed in Shiro-fugen in the greenhouse to index for ringspot, and others were placed in appropriate hosts to index for other viruses and to try establishing healthy plants from the treated buds. Inactivation of ringspot virus in the cherry buds was first detected after 17 days and in peach after 24 days.

Test trees inoculated with treated buds and bark chips from Stockton Morello and Mapes sweet cherry produced no symptoms of sour cherry yellows, prune dwarf, or peach stunt if the Shiro-fugen test was negative for ringspot. Samples taken at 17 days from these two species tested negative for all the diseases known to be present in untreated buds.

Inactivation of ringspot was not detected in Lovell peach seedlings until after 24 days of treatment, when 4 of 5 buds tested negative. All buds tested negative for ringspot after 31 days. In this case also, neither prune dwarf, sour cherry yellows, nor peach stunt were found in index trees receiving treated buds that no longer contained ringspot. As soon as the test for ringspot became negative, sour cherry yellows, prune dwarf, and peach stunt tests were also negative on Montmorency cherry, Italian prune, and peach test trees. A strain of ringspot not capable of producing sour cherry yellows, also present in the treated trees, was inactivated by the same treatment that inactivated the virus associated with sour cherry yellows, prune dwarf, and peach stunt.

Since the cultures of ringspot inactivated in sweet and sour cherry at 17 days were not inactivated in peach until 24 days, and not completely until 31 days, a host effect is suggested rather than a virus characteristic. Kassanis (3) presented somewhat different evidence that in growing plants virus inactivation may not be a direct result of heat.

New shoots produced by plants exposed to heat usually showed shock symptoms of ringspot 3–4 weeks after removal from the heated room to the greenhouse. In one case, a Stockton Morello plant heated 31 days produced shoots that showed no shock symptoms. This plant indexed negative for ringspot in subsequent years. In most cases, however, virus remained active in portions of treated plants and moved into new shoots within a few weeks after the plants were moved from the heated room to the greenhouse. Invasion of healthy tissue and production of shock symptoms after heat treatment prompted the suggestion that this phenomenon may account for the appearance of shock symptoms of ringspot in field trees during the summer in the interior valley of California (8).

The cure of ringspot, peach necrotic leafspot, Muir dwarf, and peach stunt was further investigated in several varieties of peaches in 1957 and 1958. Table 1 summarizes the results on inactivation of ringspot based on the local necrotic reaction in Shiro-fugen.

Stunting symptoms similar to those produced in peach inoculated with sour cherry yellows and prune dwarf virus cultures developed in seedling peach trees with inserted buds from Muir peach showing symptoms of Muir dwarf—in all trees with buds heated 2 weeks; in 3 of 4 trees with buds heated 3 weeks; and in none of 7 trees with buds heated 4 weeks. These data agree precisely with those obtained for ringspot inactivation (Table 1). Results with buds of the cling peach variety Peak affected by the peach stunt disease were similar. The consistent association of ringspot virus with these diseases and their similar reaction to heat are considered further evidence that these diseases are caused by the ringspot virus.

Each variety listed in Table 1 was freed of ringspot in these tests. Further, no peach necrotic leafspot symptoms have been seen in progeny trees grown from ringspot-free buds under ideal conditions for symptom expression. It is suggested that peach necrotic leafspot and ringspot in peach are caused by the same virus. The appearance of flushes of symptoms in trees during the summer could be due to periodic inactivation or immobility of the ringspot virus, followed by invasion of clean tissue produced during these periods.

Further evidence that peach necrotic leafspot is caused by the ringspot virus was obtained by indexing 27 unbudded Lovell seedlings that showed symptoms of peach necrotic leafspot (Table 2). All were positive for ringspot when indexed in Shiro-fugen. Rows of trees of varieties propagated from ringspot-free trees in an adjacent block showed about the same per cent of necrotic leafspot as the unbudded seedlings. Natural spread may have occurred, but seed transmission through the rootstock seeds is much more likely. In Table 2 are shown the results of a nursery survey on the incidence of peach necrotic leafspot in 2 peach varieties free of ringspot, in one of the varieties from

Table 2.—Occurrence of necrotic leafspot symptoms in nursery trees.

Variety	Ringspot reaction of budwood source trees	Total trees inspected	Trees showing necrotic leafspot	
			No.	%
Elberta	–	544	42	7.7
Gaum	–	900	19	2.1
Gaum	+	860	713	80.6
Lovell sdlgs. (unbudded)		900	27	3.0

a ringspot-infected source, and in unbudded Lovell seedlings. Buds of Elberta and ringspot-free Gaum were obtained from budwood source trees originating from single trees of each variety. Buds of Gaum infected with ringspot originated from a single infected tree.

Cochran (1) reported transmission of the ringspot virus through seeds of peach. His numbers were small, but the order of transmission was about the same as that shown in Table 2 for Elberta and Gaum trees on Lovell seedling rootstock and for the unbudded Lovell seedlings. Where ringspot-infected budwood of Gaum was used, the incidence of peach necrotic leafspot symptoms in the nursery trees was markedly higher. Data presented in Table 2 were obtained from a single inspection. Somewhat higher incidence of disease might have been detected if additional inspections had been made.

Wagnon et al (11) reported seed transmission of both ringspot and peach necrotic leaf-spot viruses. They found that all seedlings which showed symptoms of peach necrotic leafspot tested positive for ringspot. Some seedlings that did not show symptoms also tested positive for ringspot. These results, interpreted in light of the heat treatment results reported here, would indicate that not all cultures of ringspot virus are capable of producing summer symptoms in the field. This might be due to variation among individual seedlings, such as their tolerance to heat, or to variation of the virus. The constant association of the ringspot virus and peach necrotic leafspot symptoms in unbudded seedlings based on the Shiro-fugen test in 27 seedlings reported here and in 50 seedlings reported by Wagnon et al constitutes strong evidence that the necrotic leafspot symptoms are caused by the ringspot virus.

Conclusions.—The Shiro-fugen test (4) used for detection of the ringspot virus is assumed to be specific. No evidence to the contrary is at hand but this does not preclude the possibility that different viruses could cause the same reaction in this plant. Based on the Shiro-fugen test the ringspot virus was eliminated from propagating units by heat treatment of infected plants. This method can be used to obtain ringspot-free trees of commercial varieties.

Sour cherry yellows, prune dwarf, peach stunt, Muir dwarf, and peach necrotic leafspot symptoms were not obtained in the absence of the ringspot virus. These same diseases were invariably cured by heat treatment in the same time that was required to inactivate the ringspot virus. A strain of ringspot virus not associated with any other disease than ringspot was eliminated after the same length of treatment as those associated with sour cherry yellows, prune dwarf, peach stunt, Muir dwarf, and peach necrotic leafspot. These results are considered further evidence that strain variants of the stone fruit ringspot virus exist and cause different diseases as contended by Milbrath (6).—Department of Plant Pathology, University of California, Davis.

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PREPARING NEMATODE-FREE BANANA "SEED"

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SUMMARY

Four banana varieties cultivated for export are susceptible to *Radopholus similis*, *Meloidogyne incognita acrita*, *M. arenaria*, *Helicotylenchus erythrinae*, and *H. multicinctus*. The nematodes are distributed to new plantations on infected "seed" (rhizomes). Paring all discolored tissues from rhizomes eliminated the nematodes. Pared "seed" was surface-decontaminated in a Bordeaux-Nemagon dip and air-dried for 24 hours. Paring Gros Michel rhizomes delayed harvest of the first fruits for 2 weeks. Over a 50-week period, however, there was no significant difference between pared and regular plantings in number of harvested stems or fruit size. The beneficial effect of paring was manifested in the first ratoon crop. Up to 17 months after planting there was a 44% increase in number of harvestings and a substantial increase in fruit weight.

Introduction.—Gros Michel, Cocos, Lacatan, and Robusta bananas are the chief varieties cultivated for export in Central and South America, the West Indies, the Dominican Republic, Africa, and Australia. The Gros Michel and its Cocos sport are highly susceptible to a wilt disease caused by *Fusarium oxysporum* Schlecht. f. *cubense* (E. F. Sm.) Snyd. & Hans. Lacatan and Robusta, on the other hand, being very highly immune to the disease, have replaced Gros Michel almost completely in Jamaica (5).

Loos (4) studied the relationship between 2 plant-parasitic nematodes (*Radopholus similis* (Cobb, 1893) Thorne, 1949, and *Meloidogyne incognita acrita* Chitwood, 1949) and the fusarium wilt disease on Gros Michel. He found that prior infection with either nematode was not a prerequisite to wilt disease, though disease expression was aggravated and the period between inoculation with the fungus and the appearance of disease symptoms was considerably shortened in plants heavily infected with *R. similis*.

Plant-parasitic nematodes associated with banana "seed" (rhizomes).—All four banana varieties are highly susceptible to infection with *R. similis*. Infection causes rotting of the roots, with consequent depletion of the plant root system (4). Heavily infected plants are stunted, carry small fruit, and tip over or uproot easily because of inadequate root anchorage. The two root-knot species *M. incognita acrita* and *M. arenaria* (Neal, 1889) Chitwood, 1949, are also common pests of bananas (2, 3). The writers observed no obvious ill-effect on fruit size or plant anchorage, however, in a forty-year-old Gros Michel planting infected with *M. incognita acrita*, though in a pot experiment the same nematode stunted the early growth of Gros Michel—a symptom that disappeared as the plants aged (4). *Helicotylenchus erythrinae* (Zimmerm., 1904) Golden, 1956, *H. multicinctus* (Cobb, 1893) Golden, 1956, *Trichodorus* sp., *Hemicycliophora* sp., *Criconema* sp., *Xiphinema* sp., and *Paratylenchus* sp. are often observed in association with bananas, though there is no evidence of economic damage to the plant (2, 3).

R. similis forms lesions in roots and in the outer cortical tissues of the rhizome. Those lesions range from flecks to the black head symptom (1), involving a layer of tissue completely surrounding the rhizome. *Helicotylenchus erythrinae* and *H. multicinctus* are ecto- and endo-parasites in both roots and rhizomes, the lesions in this case being small and superficial. Infections of the root-knot nematode are confined to the roots, though galls may form close up against the rhizome at points of root emergence.

Washing the "seed" (rhizomes) and trimming off the roots reduces the potential carry-over of nematodes on the planting material, but is of limited value since the primary parasite, *R. similis*, may be found in large numbers within lesions in the rhizome.

Materials and methods.—*Seed types.*—The banana mat is an aggregate of rhizomes of varying ages and sizes. All these rhizomes may be used as "seed" for propagation, but the general farm practice is to use the larger rhizomes either as "bull-heads" (rhizomes of a recently harvested plant) or large "maiden-heads" (sucker rhizomes that have been periodically pruned and have not produced a flower). The choice of "seed" type varies between plantations and is generally guided by the size of the planting project and/or the availability of planting material. The "bull-head seed" is planted as a complete unit or is split into 2, 3, or even 4 "seed" pieces, depending on the size and number of well-developed buds it carries. The maiden-head rhizome may be similarly treated, though it is seldom split into more than 2 "seed" pieces. The smaller rhizomes, sword-suckers or "button-seed," have been successfully used for experimental work (4).

Nematode-free "seed" preparation.—Banana roots originate from the central stelar region of the rhizome and pass through the thick, fleshy cortical tissues before being externally borne. The burrowing nematode, *R. similis*, enters into the rhizome via those roots; the entrance point is clearly indicated as discolored tissue. A thin paring of the external tissues of the rhizome removes all externally borne roots and exposes the white, fleshy cortical tissues against which the conspicuous, red-fringed lesions caused by *R. similis* are clearly emphasized (Fig. 2). The exposed cortical tissues of the rhizome discolor in a few minutes and gradually become so dark that blemishes are quickly obscured. To be certain that all discolored tissue and, with it, all nematode-infected material is pared away, the paring operation must be completed before the tissues darken.

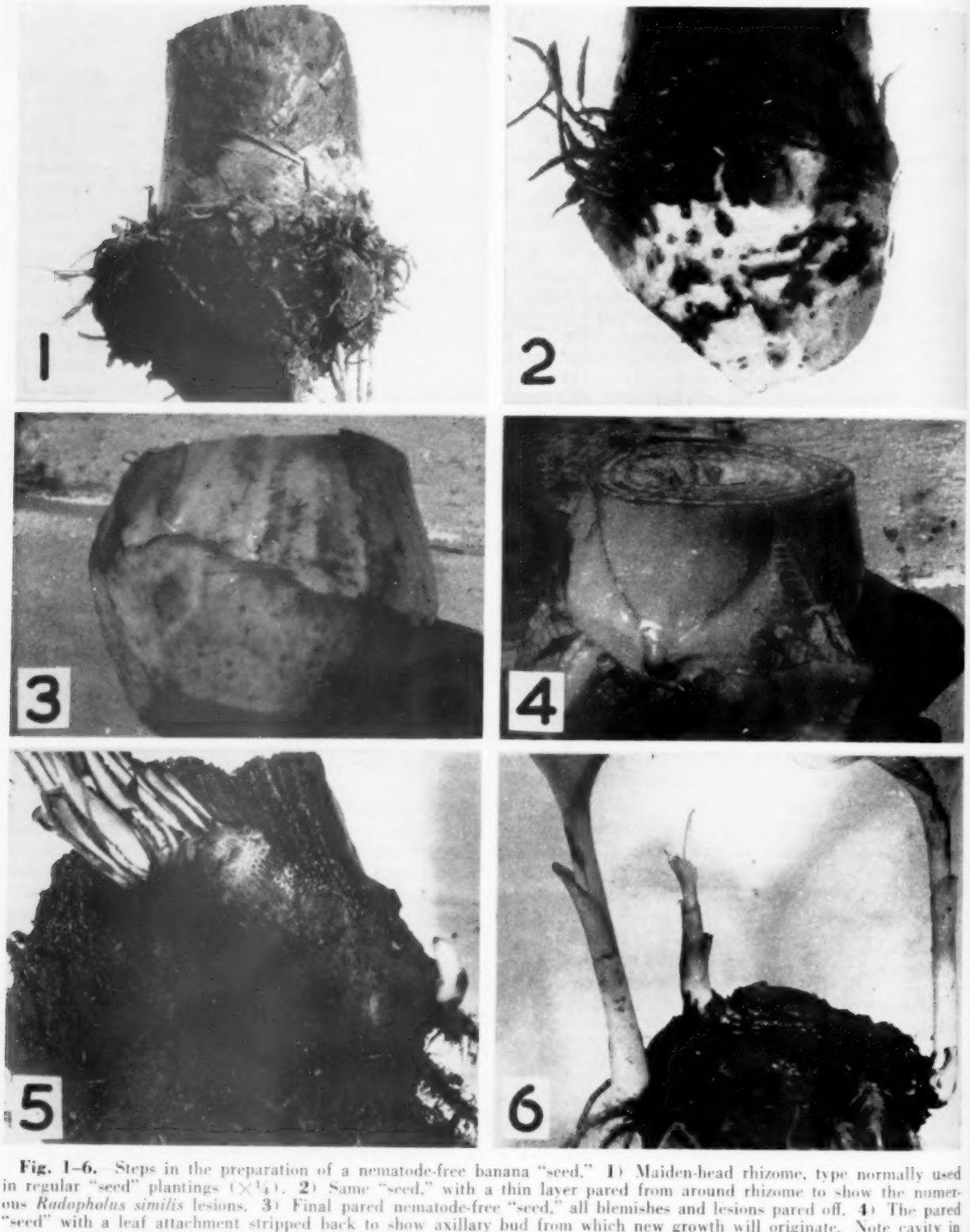

Fig. 1–6.—Steps in the preparation of a nematode-free banana "seed." **1)** Maiden-head rhizome, type normally used in regular "seed" plantings (×¼). **2)** Same "seed," with a thin layer pared from around rhizome to show the numerous *Radopholus similis* lesions. **3)** Final pared nematode-free "seed," all blemishes and lesions pared off. **4)** The pared "seed" with a leaf attachment stripped back to show axillary bud from which new growth will originate. Note cavity in center of pseudostem, formed by removal of the central heart bud. **5)** Hypertrophied growth from the remains of an imperfectly removed heart bud. Note consequent poor development of axillary bud. **6)** Seven-week-old growth from a pared nematode-free maiden-head from which the heart bud was removed.

Most "bull-heads" contain only a few pseudostem leaf attachments between which the axillary buds are located. Paring entails removal of all the large buds around the periphery of the pseudostem base and one, or possibly two, of the outer rings of those leaf attachments, making the "bull-head" unsuitable for nematode-free "seed" preparation. Large maidenheads are more suitable since the base of the pseudostem carries a number of leaf attachments (Fig. 1) and, although an outer ring with the larger axillary buds may be removed at paring, what remains is sufficiently large and active for healthy growth (Fig. 4). If the rhizome heart bud is left intact or is incompletely removed, however, growth originates at that point and the axillary buds lie dormant or make weak growth responses (Fig. 5).

A banana rhizome pared of all discolored tissue (Fig. 3) is free of both nematode and borer, *Cosmopolites sordidus* (Germ). As a further precautionary measure, in areas where fusarium and bacterium (*Pseudomonas solanacearum* E.F. Sm.) wilt diseases are known to prevail, a large section is cut off from the "seed" base to expose the stelar region in which the typical discolorations of the wilt-disease symptoms are manifested. A diseased "seed" is immediately discarded. The bacterial wilt disease organism is highly infectious and may be transmitted from "seed" to "seed" on a contaminated paring knife. To minimize this possibility, the knife should be dipped in 10% formaldehyde solution between preparation of each "seed." Should a bacterial or wilt-diseased "seed" be encountered the surface of the "seed" preparation table, the knives, and the worker's gloves are decontaminated in 10% formaldehyde before operations are resumed. The pared "seed" is finally dipped for one minute in a mixture of 44 lb lime, 44 lb copper sulphate, 12 oz Triton 100, 1288 ml of 70% Nemagon (1,2-dibromo-3-chloropropane), and 100 gal. water. The dipped "seeds" are placed in a single layer and allowed to dry under cover for at least 24 hours before being planted.

Nemagon was selected as a "seed" surface decontaminant because the formulation was not phytotoxic, even when used at double the recommended concentration. The characteristic odor of Nemagon was recognizable on the "seed" a week after it was planted, and the blue deposit from the Bordeaux dip persisted more than 10 weeks.

A shellaclike exudate gradually seeps out from the pared tissue and sets hard, in 2–3 hours, as a thin film over the rhizome. There is no indication that this exudate affords protection from rot-causing organisms. The worker manipulating the dip should wear protective gloves since the chemical preparation may otherwise cause contact dermatitis. Long cotton gloves, heavily smeared with an acetic acid petrolatum salve, fixed above the elbows and worn under heavier, wrist-high, rubberized gloves, are effective. By themselves, the rubberized gloves apparently can cause friction and unnoticeable bruising of the skin around the open end of the glove; a ring of severe dermatitis has been known to develop there.

Results.—In a pot experiment 100 small *R. similis*-infected rhizomes were pared free of nematode infections and grown 19 weeks in sterilized soil; not one plant was infected at the end of the experiment. In still another experiment over 400 "seed," pared clean of lesions on severely infected rhizomes and grown in sterilized sawdust, were nematode-free at the end of 12 weeks. Plants grown from unpared "seed," in contrast, carried severe infections.

Nematode-freed (pared) and regular (not pared) "seed" were compared in a planting in 1958 on about 450 A. of land that had been continuously under flood water for 4–6 months. The area had previously been in abandoned abaca (*Musa textilis*) infested with *R. similis* and heavily infested with *Pratylenchus musicola*. (Cobb, 1919) Filipjev, 1936 (a synonym of *P. coffeae* (Zimmerm., 1898) Goodey, 1951 (6)). The 6-month period under flood-fallowing was long enough to destroy the plant-parasitic nematodes through starvation. The area was divided into 8 fields, half of them planted with pared and the other half with regular "seed." Rhizomes for both the regular and pared "seed" preparation were obtained from the same location and planted at the same time. At the time of preparation for planting nearly 90% of the rhizome supply was found infected with *R. similis*.

Many thousands of "seed" were planted in each field. Eight months after planting, mats were removed at random and the roots and rhizomes examined for *R. similis* infection. Since the infection percentage, made on randomized samplings, of the regular "seed" plantings was so high, examinations were limited to only 25 plants in each of those fields. Larger numbers of samplings were made in the areas planted with pared "seed" (Table 1). The outstanding results are those from the comparable plantings of Fields 7 and 8. Field 7 was planted last in the series of pared "seed," by which time the labor teams were better trained and more responsible. Their experience was reflected in the 1% infection vs. a possible 89% (Table 1).

The preharvesting growth period of Gros Michel banana varies considerably between plants set out under identical conditions, whether as regular or pared "seed." In a regular vs. pared "seed" experiment, set

Table 1.—Comparison of banana plantings, from regular vs. pared "seed," for presence of *Radopholus similis*. The majority of the plantings were made with split maidenhead rhizomes weighing 6–8 lb. The numbers of mats examined, 8 months after planting, were randomized selections out of several thousand plants in each field.

Field no.	Variety	Type of "seed"	No. mats examined	% mats with *R. similis*
1	Gros Michel	Pared	1391	6%
2		Regular	25	84%
3	Cocos	Pared	746	7%
4		Regular	25	92%
5	Gros Michel	Pared	208	3%
6		Regular	25	80%
7	Gros Michel	Pared	207	1%
8		Regular	25	89%

up as 8 replicated one-acre plots of each seed type, a few of the plants in the regular "seed" plots began flowering 2 weeks before plants in the pared "seed" plots. "Seeds" planted on a single day had preharvesting periods ranging from 9 to 13 months. At the end of 50 weeks, fruit had been harvested from 35% of the regular and 30% of the pared "seed" plots, indicating that heavy paring to eliminate plant-parasitic nematodes did not delay fruiting unduly. The mean respective weights of the first 400 fruits of the pared and regular "seed" plantings were 77 and 74 lb (difference not significant). The first ratoon or the second fruit crop began 12 months after planting. Six months later, harvestings were 44% greater from the pared-"seed" plantings than from the regular-"seed" plots. The difference was attributed to burrowing nematode infestation, causing regular "seed" plants to topple or tip because of insufficient root anchorage, or, fail to reach maturity. The respective mean weights of second-crop fruit, 444 from the pared and 248 from the regular "seed" plots, were 109 and 103 lb (difference significant at the 5% level).

Discussion.—The planting of infected material has obviously been the chief means of the dispersal of the burrowing nematode on bananas. Planting material is generally obtained from plantations where the nematode is a serious pest and is present on a high percentage of the rhizomes. A large nematode population is moved with such "seed," infesting areas previously free of the pest. Thousands of acres of virgin land have thus been infested in Central and South America. In the senior writer's experience through Panama, Costa Rica, Dominica, Puerto Rico, Honduras, and Jamaica, no special nurseries are available for an extensive supply of clean planting material. The United Fruit Co. in Central America and the Banana Board in Jamaica have set up nurseries with clean "seed" for the establishment of burrowing-nematode-free plantations. "Seed" from such nurseries will aid in future expansion and eliminate or radically limit the ever-increasing radius of the present spread of the nematode pest.

It appears unlikely that the burrowing nematode will harm fruit size in the first, or plant, crop unless soil and "seed" piece are both heavily infested. During the first 6 months the nematode population builds up steadily, though root production keeps pace with root destruction. When the fruit is harvested, however, the roots of the harvested plant break down and free large populations of the burrowing nematode for invasion and destruction of the roots supporting the ratoon plants. This destruction of the root system weakens the plant's anchorage and causes it to topple or tip easily. The severe loss in harvestings of the first ratoon crop in the regular "seed" plots bears out observed losses in field plantations where the burrowing nematode is a pest. Continuity of cropping over a number of years is essential for economic returns. In the widespread occurrence of *R. similis* there is little likelihood of economic cropping beyond the second or third ratoon crops.

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THE INFLUENCE OF TEMPERATURE ON DEVELOPMENT OF PHYTOPHTHORA PARASITICA ROOT ROT OF FUCHSIA

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SUMMARY

In a root rot of fuchsias observed near Portland, Oregon, in 1958, plants of various ages and sizes wilted, and died a few days after symptoms first appeared. Isolations from affected plants consistently yielded *Phytophthora parasitica*. Isolates were parasitic on fuchsia and caused a root rot disease. Temperature was the limiting factor in the disease syndrome. Plants at 85°F were infected within 24 hours of inoculation and began to wilt after 7 days. Plants kept at 65°F and 75°F became infected but did not show disease symptoms. Transfer of inoculated plants to 75°F after 48 hours at 85°F stopped disease development. After 80 hours at 85°F, the disease had developed to such an extent that transferring plants to 75°F did not prevent symptom development and death. The outbreak of this disease in 1958 was attributed to development of latent infections because of the unusually high summer temperatures.

Introduction.—In the Pacific Northwest, several varieties of *Fuchsia* sp. are grown in gardens, greenhouses, and lath houses. In July, 1958, reports were received of a serious disease of fuchsias in a greenhouse and lath house in the Portland, Oregon, area. Affected plants wilted suddenly, and died within a few days. Isolations made from diseased tissues consistently yielded a white phycomycetous fungus subsequently

identified as *Phytophthora parasitica* Dastur (1, 4). Prior to 1958 there had been no reports of such a disease on fuchsias, although Frezzi (2) isolated *P. parasitica* from fuchsia plants. The widespread loss of plants, even old stock plants in hanging baskets, in the summer months of 1958 indicated that the plants might have been infected for some time but that conditions had not favored disease development. Studies were conducted to determine the cause of root rot of fuchsias and the influence of soil temperature on disease development.

Methods and materials.—Isolations from infected roots and stems were made by surface-sterilizing portions of plants one minute in 10% Clorox (5.25% sodium hypochlorite), then transferring sections of diseased tissue directly to Petri plates of potato-dextrose agar (PDA) containing 50 ppm streptomycin nitrate.

Inoculum was prepared by growing the fungus in pea broth at 30°C. Petri plates, each containing 15 ml of pea broth, were inoculated with *P. parasitica* and incubated at 30°C. A number of small colonies appeared after 24 hours of incubation. After 36 hours the colonies were washed twice with sterile distilled water, covered with distilled water, and incubated 24 hours at 25°C. This procedure resulted in abundant production of sporangia from which hundreds of zoospores developed. The contents of all Petri plates were transferred to an Erlenmeyer flask, and the mycelial colonies were macerated slightly with a glass rod. This suspension was used as the inoculum.

Plants used for experiments were approximately 3-month-old fuchsias grown from cuttings. These plants were grown in clay pots containing a 1:3 mixture of peat and a light sandy loam soil. Temperature tank cans were covered inside and outside with polyethylene sacks and were filled ¾ full with sand, which was used as a heating medium. Clay pots with plants were then placed in the sand with the tops of the pots just above the sand level.

Inoculations were made by boring 2 holes with a cork borer in soil on opposite sides of each plant and near the edge of the clay pots and then pouring 10 cc of inoculum into each hole. The holes were filled with soil and the pots were watered thoroughly. Uninoculated controls were treated similarly except that sterile water was poured in the holes.

Identification of causal organism.—The fungus isolated from and found pathogenic to fuchsia plants was identified by using Frezzi's (2), Leonian's (3), and Tucker's (5) keys and by comparison with a known culture of *Phytophthora parasitica* with respect to rate of growth, gross morphology, and the size and shape of sporangia. Since all attempts to produce oospores of the fuchsia pathogen failed, criteria other than the ones based on oospores were used.

The fuchsia isolates made widely spreading growth on malt extract agar and profuse growth on corn meal agar, but failed to produce oospores on lima-bean, potato-dextrose, corn meal or oatmeal agar. The isolates grew at 35°C on corn-meal, and other media, and grew in the presence of malachite green at a concentration of 1:4,000,000. Sporangia were papillate. These characteristics are typical of *P. parasitica*.

Pathogenicity trials.—To ascertain whether *Phytophthora parasitica* was pathogenic to fuchsia plants, pathogenicity experiments were conducted in temperature tanks held at different temperatures. Eighteen plants were inoculated. Six inoculated plants and 6 control plants were placed in each of 3 temperature tanks maintained at 65, 75, and 85°F.

Seven days after inoculation all 6 inoculated plants in the 85°F temperature tank began to wilt. Symptoms were similar to those observed on plants in a greenhouse near Portland. Within 4–5 days after wilting began the plants had dried completely and were dead. Inoculated plants held at 65 and 75°F and control plants at all temperatures were apparently healthy. Isolations from the inoculated plants kept at 75°F were negative during the first week after inoculation, but after 10–14 days *Phytophthora parasitica* was recovered. After 22 days the inoculated plants kept at 65° and 75°F still showed no symptoms of disease.

These plants were then divided into 2 groups. Three inoculated plants and 3 controls from the 65°F series, and a similar group from the 75°F series were transferred to 85°F and watered. The remaining plants in 65 and 75°F temperature tanks were left in the respective tanks to use in repeating the experiment.

Inoculated plants began to show typical disease symptoms on the 16th day after transfer to the 85°F temperature tank. No differences were noticed in time or nature of symptom production between plants transferred from 65°F and those transferred from 75°F. Within about 10 hours of appearance of disease symptoms 3 of the plants showing symptoms were transferred to the 75°F temperature tank to see whether they would recover following transfer to a lower temperature within a few hours of symptom appearance. None recovered. The results obtained in this experiment were duplicated by using the other plants remaining at 65 and 75°F.

Influence of temperature.—Another experiment was conducted to ascertain whether high temperature influences infection principally, or both infection and disease development. Twenty-one plants were inoculated and kept at 85°F, and 5 control plants were maintained at the same temperature. Twenty-four hours after inoculation, 6 plants were taken out of the 85°F temperature tank. Three of these plants were put in a 75°F temperature tank, and 3 were taken out of the clay pots, washed, and brought to the laboratory for isolations. A similar procedure was followed after 48 and 80 hours.

To avoid the possibility of false results from germination of zoospores on root surfaces, the entire plants were sterilized 15 minutes in 10% Clorox. This ensured isolation of the fungus from roots only if it had penetrated the tissue. From each plant, 3 long roots having secondary and tertiary branches with dark-colored areas on them, were selected. These were surface-sterilized, cut into pieces with a sterile razor blade, plated on PDA, and incubated 12 hours at 30°C. Isola-

Table 1.—Temperature data for recent summers recorded by the U. S. Weather Bureau at the Portland Airport Station.

Year	Month	Av. (°F)	Highest (°F)	No. days 90°F or above
1955	June	60.7	95	2
	July	63.0	86	0
	August	65.5	90	1
1956	June	59.0	81	0
	July	68.0	102	4
	August	65.4	93	1
1957	June	62.2	79	0
	July	65.6	87	0
	August	66.6	86	0
1958	June	65.2	95	4
	July	70.0	102	8
	August	70.0	95	3

tions showed that infection had occurred regardless of whether the isolations were made 24, 48, or 80 hours after inoculation.

When plants were transferred from 85°F to 75°F at intervals of 24 or 48 hours after inoculation they developed no symptoms. Plants left at 85°F for 80 hours after inoculation and then transferred to 75°F began to show symptoms on the 7th day after inoculation. Symptom development coincided exactly with that on the plants left in the 85°F temperature tank. This showed that in plants at 85°F the disease developed in 80 hours to such an extent that transfer of the plants to a lower temperature (75°F) did not stop disease development.

Discussion.—The studies on pathogenicity show that *P. parasitica* is a cause of fuchsia root rot and that temperature is the principal limiting factor in development of the disease. When inoculated plants were incubated at 65°, 75°, and 85°F, the plants at 85°F were the only ones that developed disease symptoms.

Inoculated plants kept at 85°F for 24, 48, and 80 hours were infected but the disease did not develop if the plants were transferred to a 75°F temperature tank after 24 or 48 hours. Although infection occurred within the first 24 hours, only the 80-hour period proved enough for the fungus to develop in the plant to such an extent that transfer to lower temperature did not prevent disease development and death of the plants. Thus, sustained high temperature is very important in the development of fuchsia root rot.

Isolations from inoculated plants kept at 75°F showed that they became infected within 10–14 days, although no symptoms developed. Thus, infection may occur at lower temperatures but disease development requires higher temperatures later. High temperatures speed up infection and favor disease development in infected plants.

Since temperature was so critical, weather data for the past 4 years were studied in an effort to explain the outbreak of fuchsia root rot in 1958. The widespread outbreak of disease of young plants and in old stock plants, many in hanging baskets and so located that introduction of inoculum would be difficult, indicated that some, if not most, of the plants had latent infections, or that the soil was infested. Normal summer temperatures in the Willamette Valley might permit infection but would not be high enough to favor disease development. Therefore a high percentage of plants in a planting might be infected without any obvious development of disease. Temperature data (6) show almost twice as many hot days in 1958 as in the previous 3 years combined (Table 1).

Temperatures in the Pacific Northwest fluctuate widely during day and night, and it is very hard to interpret weather data in relation to results obtained in the greenhouse under controlled temperature conditions. Since the average summer temperatures in 1958 were higher than in previous years, however, and since there were many more days with temperatures above 90°F, it is likely that the high temperatures in 1958 were responsible for disease development. Soil temperature does not fluctuate as much as the atmospheric temperature, and the frequency of high temperatures in 1958 may have kept soil temperatures high for a period long enough to favor disease development. Disease development may have been favored also by a cumulative effect of high temperatures. It is also possible that a few plants may have been diseased in years prior to 1958 but were not detected or reported.

These results may also explain other sporadic outbreaks of root diseases caused in particular by *Phytophthora* species.—Department of Botany and Plant Pathology, Oregon State College, Corvallis, Oregon.

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RADISH YELLOWS, A DISEASE OF RADISH, SUGAR BEET, AND OTHER CROPS

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SUMMARY

Radish yellows, a recently discovered virus disease, is characterized by yellowing of the foliage of sugar beet, spinach, lettuce, radish, and certain other crop and weed plants in California. Incidence is high in sugar beet, spinach, and lettuce and it may reduce yields in these crops. The virus is apparently unrelated to the sugar beet yellows or malva yellows viruses. Species infected by the radish yellows virus showed, in general, interveinal yellowing of the lower and intermediate leaves. Symptoms on sugar beet were indistinguishable from those induced by milder isolates of the beet yellows virus. Radish yellows is incited by a persistent aphid-transmitted virus. Vectors are *Aphis helichrysi*, *Macrosiphum dirhodum*, *M. granarium*, *Myzus ornatus*, and *M. persicae*. *Myzus persicae* is an efficient vector of the virus. The virus was acquired by nonviruliferous aphids in a 5-minute acquisition feeding period. It was transmitted to healthy plants by viruliferous vectors in a 10-minute feeding period. The incubation period of the virus in the vector was between 12 and 24 hours. Insects reared on diseased plants retained the virus up to 29 days when transferred daily on seedling plants.

Mild yellowing diseases of sugar beet have been mentioned from England (10, 17), Ireland (4), Australia (12), and recently from the United States (5). The viruses responsible for the European and Australian diseases have been distinguished from the beet yellows virus by symptom and serological responses. The mild yellowing virus of sugar beet and other plants described in California has been distinguished from the beet yellows virus on the basis of vector relationships and host range.

Studies of yellows type viruses isolated from sugar beet and other crop plants and weed hosts have distinguished a component of the yellowing complex of sugar beet, designated herein as "radish yellows virus," a typically persistent aphid-transmitted virus. It is distinguished from the beet and malva yellows viruses on the basis of vector and host relationships and is apparently unrelated to either.

Materials and methods.—Radish yellows virus was obtained from sugar beet (*Beta vulgaris* L.), spinach (*Spinacea oleracea* L.), radish (*Raphanus sativus* L.), and other hosts collected in California. Differences in severity were common between isolates collected from various sources. The possibility that some of these isolates represented strains of the radish yellows virus, and that others were distinct virus entities, led to the study of host range and vector relationships with single isolates. In the studies, an isolate originally from radish was used.

Nonviruliferous green peach aphids (*Myzus persicae* (Sulz.)) were reared on radish plants tested at frequent intervals for the radish and malva yellows viruses. The other species of aphids used were reared on appropriate host plants in an insectary. In routine transmission tests, the insects were permitted to feed on the virus source for 48 hours and then for an equal period on the test plants. Small glass insect cages were used to confine aphids on individual seedlings in pots. Recovery tests were carried out in cloth cages large enough to contain six 6-in. pots.

Geographical distribution.—The radish yellows virus is very prevalent in weed and crop plants in the San Joaquin and Salinas Valleys of California. It was found in all seasons of the year on susceptible host plants.

Nothing is known of the occurrence of radish yellows in other parts of the United States or in other parts of the world, although host plants are widely distributed.

Economic importance.—The economic importance of the radish yellows virus, although not fully ascertained, seems to be significant. Limited indexings of plants in the Salinas and San Joaquin Valleys have indicated a high incidence of this virus, alone or accompanied by the beet yellows virus in yellowed sugar beets. In some instances, this entity in beets appeared to be more prevalent than the beet yellows virus.

In the Salinas Valley, the virus was isolated in high incidence alone or in combination with the malva and beet yellows viruses from spinach plantings in which the plants were severely stunted and yellowed.

It has been isolated alone or in combination with the malva yellows virus from lettuce fields showing large amounts of foliage yellowing (6).

The radish yellows virus was isolated from a high percentage of plants tested from radish seed fields showing foliage yellowing.

In the greenhouse, the virus causes a yellows disease of pea (*Pisum sativum* L.), flax (*Linum usitatissimum* L.), broccoli (*Brassica oleracea* L. var. *botrytis* L.), turnip (*Brassica rapa* L.), watermelon (*Citrullus vulgaris* Schrad.), and ornamentals such as zinnia (*Zinnia elegans* Jacq.), petunia (*Petunia hybrida* Vilm.), and mignonette (*Reseda odorata* L.), and possibly may occur in these species in nature.

Host range.—Host-range studies were carried out by 2 methods. Recovery tests from field plants showing yellowing gave evidence as to some of the species naturally infected with the radish yellows virus. The virus was recovered by feeding nonviruliferous green peach aphids on collected field plants for 48 hours and transferring the insects to healthy indicator seedlings for 48 hours.

Bennett (1) and Bennett and Costa (2) have shown that *Chenopodium amaranticolor* Coste & Reyn. and *C. capitatum* (L.) Asch. produce distinct symptoms when infected with strains of the beet yellows virus. Bennett indicated that shepherd's-purse (*Capsella bursa-pastoris* (L.) Medic.) is apparently not susceptible to the known American strains of the beet yellows virus. Costa, Duffus, and Bardin (5) have shown that

cheeseweed (*Malva parviflora* L.) is a good indicator host of the malva yellows virus.

The indicator plants used in these tests included sugar beet, shepherd's-purse, cheeseweed, *Chenopodium amaranticolor*, *C. capitatum*, and radish. The aphids placed on the radish plants were allowed to remain for 7 days, after which they were transferred to a new set of indicator hosts. Through use of this technique, the beet yellows, malva yellows, and radish yellows viruses were readily separated and identified. The beet yellows virus gave a positive reaction on sugar beet, *C. amaranticolor*, and *C. capitatum*, but no reaction on shepherd's-purse and cheeseweed. Also, the beet yellows virus did not survive within the vector during the 7 days on radish. The malva yellows virus gave a positive reaction on cheeseweed and shepherd's-purse but no reaction on the other species. It survived 7 days in the vector on radish, and subsequently infected cheeseweed and shepherd's-purse. The malva yellows virus, in these tests, could occasionally be recovered from the inoculated sugar beets, which did not show symptoms under greenhouse conditions. The radish yellows virus produced a positive reaction on sugar beet and shepherd's-purse and survived 7 days in the vector on radish.

Additional host-range studies were carried out by inoculation of at least 5 seedlings of a number of different species in an insectary compartment, where a large population of viruliferous green peach aphids was being reared on diseased radish. A large number of insects were shaken from the virus source plants onto those being inoculated, and were permitted to feed for at least 48 hours. Presence or absence of virus in each of the plant species tested for susceptibility was determined by aphid transfer to shepherd's-purse seedlings about 60 days after inoculation. From recovery tests from field and greenhouse plants, host plants of the radish yellows virus are as follows: **Amaranthaceae.**—*Amaranthus retroflexus* L., *A. tricolor* L., *Celosia argentea* L. **Caryophyllaceae.**—*Cerastium viscosum* L., *Spergula arvensis* L., *Spergularia rubra* (L.) J. & C. Presl., *Stellaria media* (L.) Cyr. **Chenopodiaceae.**—*Beta macrocarpa* Guss., *B. patellaris Moq.*, *B.* sp (annual from Turkey), *B. vulgaris* L., *B. vulgaris* L. var. *cicla* L., *Spinacia oleracea* L. **Compositae.**—*Lactuca sativa* L., *L. serriola* L., *Verbesina encelioides* (Cav.) Benth. & Hook., *Zinnia elegans* Jacq. **Cruciferae.**—*Brassica oleracea* L. var. *botrytis* L., *B. rapa* L., *Capsella bursa-pastoris* (L.) Medic., *Raphanus sativus* L. **Cucurbitaceae.**—*Citrullus vulgaris* Schrad. **Geraniaceae.**—*Erodium cicutarium* (L.) L'Her., *Geranium dissectum* L. **Leguminosae.**—*Pisum sativum* L., *Trifolium alexandrinum* L. **Linaceae.**—*Linum grandiflorum* Desf., *L. usitatissimum* L. **Resedaceae.**—*Reseda odorata* L. **Solanaceae.**—*Nicandra physalodes* (L.) Gaertn., *Nicotiana bigelovii* S. Wats., *N. clevelandii* Gray, *Petunia hybrida* Vilm. This list shows that a number of economic plants and weed species are hosts of this virus.

Recovery tests from field sugar beets.—Limited recovery tests from yellowed field sugar beets in the Salinas and San Joaquin Valleys were conducted with the technique described. Of 50 yellowed sugar beets selected at random from 5 different fields in these areas, 76% had the radish yellows virus alone, 4% the beet yellows virus alone, and 12% both viruses.

Symptoms.—Species infected by the radish yellows virus showed, in general, yellowing of the lower and intermediate leaves (Fig. 1). Some species, however, exhibited a general chlorosis, and others were symptomless carriers. Symptoms on a selected group of host plants follow:

Beta vulgaris.—Greenhouse-inoculated sugar beet plants showed initial symptoms in 12–35 days. These were usually a mild chlorotic spotting of the interveinal areas on the older and intermediate leaves. As the disease progressed, the yellowing became more intense and involved more of the interveinal tissue. Yellowed portions were at times sharply delimited by the veins. Older infected leaves were yellow except for green areas adjacent to the veins and became thickened and brittle.

In adjacent plants, previously inoculated with mild isolates of the beet yellows and radish yellows viruses, it was impossible to distinguish the 2 viruses on the basis of symptoms on sugar beet.

Various isolates from field-infected plants differed slightly in their symptom syndrome and severity. In several instances, these isolates were shown to be fairly stable and may represent strains or separate virus entities.

Spinacia oleracea.—Spinach plants infected with the radish yellows virus in the greenhouse showed mild yellowing symptoms in the interveinal areas and near the leaf tips.

Nicotiana clevelandii.—Chlorotic blotching of the older leaves occurred within about 15 days after inoculation. As the plants aged the lower leaves became completely chlorotic and the upper ones showed marked interveinal chlorosis.

Lactuca sativa.—Greenhouse-infected lettuce plants exhibited, at first, irregular chlorotic blotching, sometimes delimited by the veins, on the older and intermediate leaves. Later the chlorotic areas tended to coalesce and produced severe interveinal yellowing symptoms, especially near the leaf margins and base. These leaves later turned almost completely chlorotic, except for narrow bands along the main veins.

Capsella bursa-pastoris. — Greenhouse-inoculated shepherd's-purse plants showed initial symptoms 10–20 days after inoculation. Lower leaves developed severe chlorosis and moderate leaf curl. As the disease progressed, the yellowing developed acropetally. Yellow leaves were thickened and brittle. The plants were markedly stunted.

Nicandra physalodes.—Scattered chlorotic blotches occurred on the lower leaves of inoculated plants 12–22 days after inoculation. Later, the yellowed areas coalesced and formed large interveinal chlorotic areas that at times were sharply delimited by the veins. Still later, these leaves turned completely chlorotic, except

for narrow bands adjacent to the veins.

Transmission tests.—*Seed.*—Preliminary tests to determine whether the radish yellows virus is transmitted through the seed of infected hosts were carried out with shepherd's-purse and lettuce. Several thousand seedlings were grown in flats from seeds of shepherd's-purse produced by diseased field plants and about a thousand seedlings from seeds of lettuce plants infected in the greenhouse. Careful observations for about 2 months failed to show any evidence of radish yellows infection.

Mechanical.—Several attempts were made to transmit the radish yellows virus mechanically, by routine techniques that included the use of abrasives, phosphate buffer, and sodium sulphite. The virus sources used in these tests were sugar beet, shepherd's-purse, and *Nicotiana clevelandii*. The plants inoculated included these and a number of other species found to be susceptible when inoculated by the aphid vector.

In all but one test, the results were negative. In the positive test, 24 sugar beet and 28 shepherd's-purse plants were inoculated with a radish yellows isolate from shepherd's-purse. The inoculum was extracted in the presence of a mixture of 0.02*M* phosphate buffer at pH 7 and 0.02*M* sodium sulphite. Three shepherd's-purse plants showed typical radish yellows symptoms, and subsequent testing with the aphid vector confirmed the presence of the virus. Subsequent mechanical inoculations from the same source and from the plants infected in this test gave negative results. The possi-

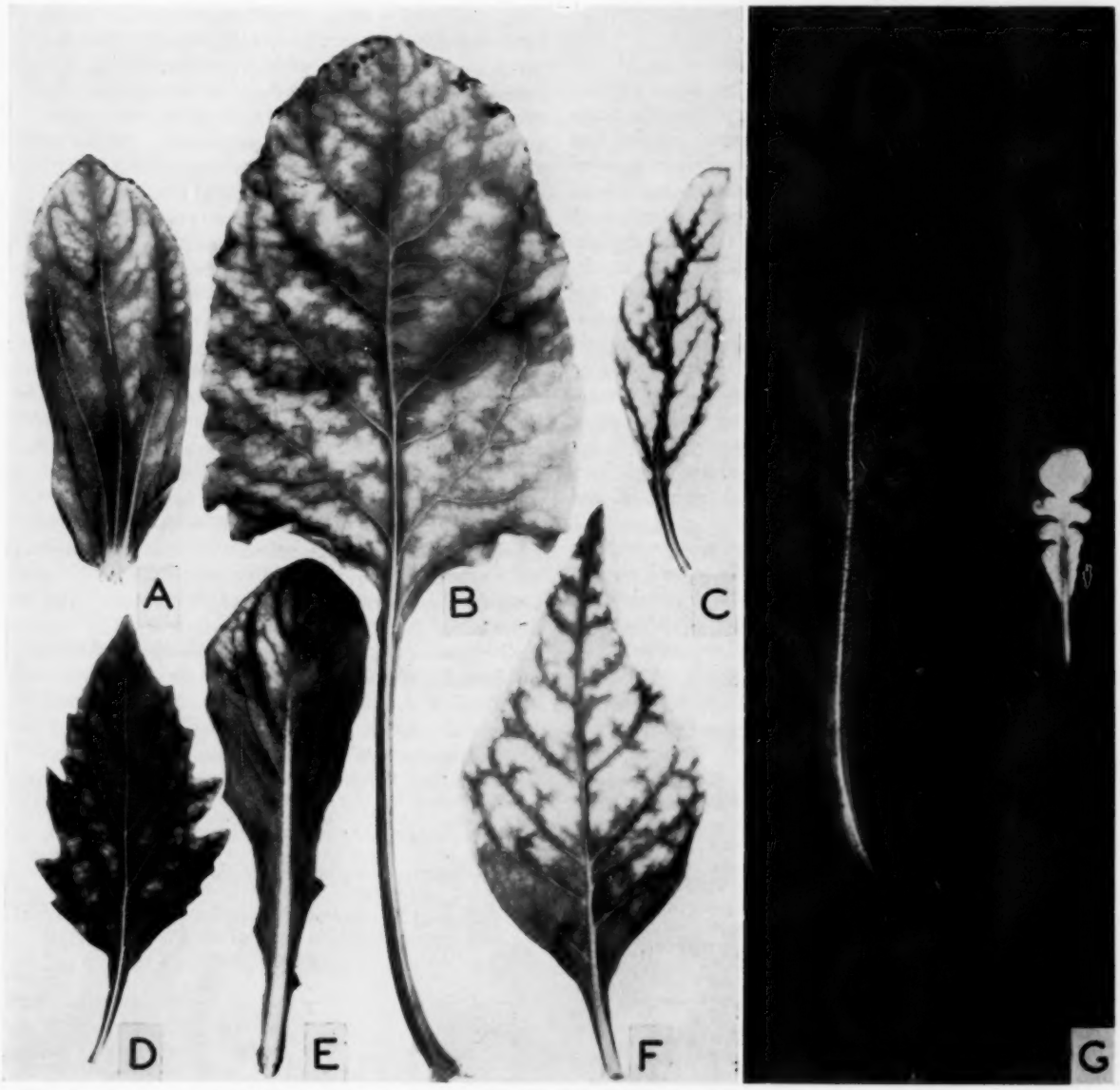

Fig. 1.—Leaves of greenhouse plants showing symptoms induced by the radish yellows virus. **A)** *Zinnia elegans.* **B)** *Beta vulgaris.* **C)** *Verbesina encelioides.* **D)** *Nicandra physalodes.* **E)** *Lactuca sativa.* **F)** *Nicotiana clevelandii.* **G)** *Capsella bursa-pastoris;* left, healthy leaf.

Table 1.—Relation of numbers of green peach aphids to transmission of the radish yellows virus.

Test no.	Number of shepherd's-purse seedlings infected out of 8 inoculated when colonized with the indicated number of viruliferous insects per plant			
	1	3	9	27
1	4	4	6	7
2	1	3	5	4
3	3	6	8	8
4	6	8	8	8
5	1	4	4	7
6	5	6	8	8
% transmission	41.7	61.6	81.3	87.5

bility exists that infection resulted from a stray viruliferous vector, though contaminations were not evident in the other experiments.

Insects.—Tests to determine whether some of the common aphid species are vectors of the radish yellows virus were carried out with radish or shepherd's-purse as the virus source and shepherd's-purse as the test plant. Nonviruliferous aphids of the various species tested were placed on the source plants for 24–48 hours, and then about 25 individuals were transferred to each of a number of test plants for an infection feeding period of about 48 hours. Under these conditions, the following aphid species transmitted the radish yellows virus: *Aphis helichrysi* Kalt., *Macrosiphum dirhodum* (Walker), *Macrosiphum granarium* (Kirby), *Myzus ornatus* Laing. and *Myzus persicae* (Sulz.). Of these, only the last 2 have been shown capable of transmitting beet yellows and malva yellows viruses (1, 5).

Virus-vector relationships.—Virus-vector relations were studied only for the green peach aphid, *Myzus persicae*.

1) *Relation of numbers of insects to virus transmission.*—Viruliferous green peach aphids reared on diseased radish plants were used in tests to determine the relative efficiency of different numbers of insects in securing infection with the radish yellows virus. The insects, singly or in groups of 3, 9, or 27, were given a 48-hour infection feeding period on shepherd's-purse test plants. The results (Table 1) indicate that single insects are capable of transmitting the virus to more than 40% of the test plants. In other tests, in relation to the persistence of the virus within the vector, 70% of some 40 insects transferred serially induced infection in 1 or more test plants.

2) *Acquisition feeding period.*—The feeding time on a virus source plant required for nonviruliferous green peach aphids to become infective was studied over a feeding period range of 5 minutes to 48 hours. After the feeding period on diseased radish, the insects were removed and placed in groups of 5 on shepherd's-purse seedlings for a 48-hour infection feeding. The results (Table 2) indicate that the insects may become viruliferous in a 5-minute feeding period. The efficiency of the vectors increased greatly with an increase in feeding time on the virus source.

3) *Infection feeding period.*—Tests to determine the time required for viruliferous aphids to transmit the virus were carried out with insects reared on diseased radish using feeding periods of 5–90 minutes. Groups of 5 insects were placed on each of the test plants and permitted to feed for different periods. At the end of the feeding period, the insects were removed and the plants were sprayed with TEPP. The results (Table 3) indicate that viruliferous green peach aphids were capable of transmitting the virus in 10 minutes. The longer the feeding time the higher the transmission percentage.

4) *Incubation period of the virus in the vector.*—Incubation period of the radish yellows virus in the vector was studied by allowing nonviruliferous aphids to feed on a virus source for 3 or 6 hours and then transferring them in groups of 5 per plant to healthy shepherd's-purse for the necessary time intervals to permit testing for incubation periods of 6, 12, 24, 48, and 96 hours. The results (Table 4) indicate that the incubation period of the radish yellows virus in the green peach aphid was between 12 and 24 hours.

5) *Persistence*—The ability of viruliferous green peach aphids to retain the radish yellows virus was determined by 2 methods. Aphids reared on diseased radish plants were used singly in daily serial transfers on healthy shepherd's-purse seedlings. The results obtained with 10 of the aphids that survived the longest are shown in Table 5. The insects were highly efficient in transmitting the virus during the first 20 transfers, but there was a definite loss in transmitting

Table 2.—Results of tests to determine the time required for nonviruliferous green peach aphids to become infective with the radish yellows virus.

Test no.	Number of shepherd's-purse seedlings infected out of 8 inoculated with groups of 5 insects fed on the virus source for the indicated period						
	Minutes					Hours	
	5	10	20	45	90	24	48
1	1	1	0	0	2	5	7
2	0	0	0	0	1	5	6
3	0	3	0	0	1	6	6
4	0	0	0	0	1	8	8
5	0	0	0	0	0	1	8
6	0	0	0	0	3	7	8
% transmission	1.6	8.3	0	0	16.7	66.7	89.6

Table 3.—Results of tests to determine the feeding time required by 5 viruliferous green peach aphids to infect shepherd's-purse seedlings with the radish yellows virus.

Test no.	Number of plants infected out of 8 on which the aphids were allowed to feed for the indicated period in minutes				
	5	10	20	45	90
1	0	0	1	2	1
2	0	0	2	3	4
3	0	1	2	5	5
4	0	0	0	0	3
5	0	5	3	2	6
6	0	0	0	2	0
% transmission	0	12.5	16.7	29.2	39.6

Table 4.—Results of tests to determine the incubation period of the radish yellows virus in the green peach aphid.

Test no.	Feeding period on virus source in hours	Number of shepherd's-purse seedlings infected out of 8 inoculated with 5 insects in which the virus had the following incubation period in hours				
		6	12	24	48	96
1	3	0	0	1	2	1
	6		0	0	1	5
2	3	0	0	0	4	2
	6		0	0	5	5
3	3	0	0	1	0	1
	6		0	0	4	2

ability in the later transfers.

Most of the insects that acquired the virus remained viruliferous throughout their life. This is an apparent distinction between the radish and malva yellows viruses. The latter was not retained as long.

The ability of viruliferous aphids to retain the radish yellows virus when feeding on an immune host was determined by placing insects reared on diseased radish on immune tomato (*Lycopersicon esculentum* Mill.) plants and then testing the aphids at intervals by transferring them to shepherd's-purse seedlings. Under these conditions, the virus was retained in the vector up to 9 days, the longest period the insects survived on tomato. The tomato plants were tested at the termination of the tests and found to be virus free.

Discussion.—The radish yellows virus differs from the malva yellows virus on the basis of a number of different host reactions. The 2 entities also differ slightly in their vector relationships. Both entities are highly persistent in the aphid vector, and are readily distinguished from the beet yellows virus on this basis, the beet yellows virus being semi-persistent and retained for a much shorter period (1, 13, 15, 16). The radish yellows virus is also readily distinguished from the beet yellows virus by host reactions. All isolates of the beet yellows virus obtained from sugar beet fields in California, during these tests, produced a positive reaction on *Chenopodium amaranticolor* and *C. capitatum*, but were negative on *Capsella bursa-pastoris* plants, whereas all radish yellows isolates were negative on the 2 Chenopodium species but produced marked symptoms on Capsella. These host reactions were tested repeatedly by attempted recovery from the symptomless plants, but in every instance the results were as reported. Several European reports (3, 11) have mentioned the use of *Capsella bursa-pastoris* as a test plant for the beet yellows virus and as a host that may give rise to attenuated virus. It is a definite possibility that the radish yellows virus may occur in Europe on beet and other crop plants and that its reactions on Capsella and other host plants may be confused with those of the beet yellows virus. Of course, the possibility exists that the common beet yellows virus isolates in California have a host range different from that of the common European isolates.

The radish yellows virus could readily occur as a contaminant in the aphid colonies grown on radish for virus transmission studies and may be important from that aspect.

There are several virus entities reported in the literature that could be related to the radish yellows virus. Among these are "the Irish Mild Strain," "41 Yellows Virus," and "the sugar beet mild yellowing virus," recently reported to be in high incidence in England, intermixed with beet yellows (10). These entities differ from beet yellows on the basis of a lack of serological response. The interrelationships of these 3 European entities are not known.

A report from Canada (7) mentioned a latent persistent virus of turnip. This same entity is thought to occur in Maine (18). These have been reported as inducing no symptoms on sugar beet; however, certain mild isolates of the radish yellows virus produce extremely mild symptoms on this host.

A yellowing disease of turnip, which also may be related to the radish yellows virus (14), has been mentioned from Belgium.

Yellowing diseases of flax (8), pea (9), and other hosts of the radish yellows virus have been reported from various parts of the world. The possibility exists that this entity may be worldwide in distribution and that its effects have been confused with nutritional and other disorders.

The implication of the widespread occurrence of the radish yellows virus in California sugar beet plantings, coupled with recent findings indicating reductions of

Table 5.—Shepherd's-purse seedlings infected (+) and uninfected (–) in daily serial transfers using single apterous green peach aphids reared on a radish yellows source plant.

Aphid no.	Successive daily transfers																														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	–	–	+	–	–	–	+	–	–	+	–	–	d*
2	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	–	–	–	+	+	–	–	d
3	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	–	+	–	–	–	–	–	+	–	–	–	d		
4	–	+	+	+	+	+	–	–	–	–	–	–	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	d				
5	–	+	+	+	+	+	–	+	+	–	+	+	+	+	–	–	+	–	+	+	+	–	–	–	–	–	d						
6	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+	–	+	+	–	d							
7	+	+	+	+	+	+	+	+	+	+	+	+	d																				
8	+	+	+	+	+	–	+	+	+	+	+	+	d																				
9	+	+	+	+	+	–	–	+	+	+	+	d																					
10	+	+	+	+	+	+	–	–	d																								

* Insect died.

up to 19% in sugar yield due to infection by an isolate of this virus, is the possible need for the incorporation of resistance studies to this entity as well as the beet yellows virus in a breeding program.—U. S. Agricultural Research Station, Salinas, California.

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BOTRYOSPHAERIA RIBIS AND ITS RELATION TO A ROT OF APPLES

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SUMMARY

Isolates of *Botryosphaeria ribis* from 8 different host species and 9 geographical locations were studied in pure culture and tested for their ability to induce a rot of apples. Cultures derived from single conidia produced in the same pycnidium were indistinguishable in cultural characteristics. Conidial lines obtained from different pycnidia, not produced in close association, were frequently distinguished on the basis of cultural characteristics. These differences in characteristics were noted between mass isolates obtained from different host species, from different parts of the same host plant, and from plants of the same or different host species not grown in the same geographical location. Characteristics most sharply defined were mycelial coloration, size of conidia, and chromogenesis on alkaline starchy media. All but one isolate studied induced a rot of apple. At temperatures of 65 or 75°F inoculated apples developed rot symptoms rapidly in most cases. Under these conditions the rot was characteristically soft in texture and clear or colorless to pink-tan in color. Temperatures of 85 and 55°F were generally less favorable for rot development.

The ascomycetous fungus *Botryosphaeria ribis* Gross. & Dugg. was originally described (5) as a pathogen of currant, and later as the causal agent of a rot (3) and canker (12) of apple. The fungus has been reported in the United States on more than 84 species of higher plants. It has also been reported on many of these and other woody species throughout the principal agricultural areas of the world.

The disease of apple fruit incited by *B. ribis* has been called "bot rot," "white rot," "clear rot," and "Botryosphaeria rot." Symptomatology and epiphytology of bot rot and black rot (*Physalospora obtusa* (Schw.) Cooke) are strikingly similar. Variation in bot rot symptoms have been attributed in part to inherent factors in isolates of *B. ribis* (3). A characteristic chromogenesis of isolates of *B. ribis* grown on starchy media (5) has been used to differentiate morphologically indistinguishable forms of the fungus. The achromogenic isolates also were reportedly nonpathogenic (5). Several asexual fruiting stages and spore types have been ascribed (5) to *B. ribis*.

Studies were made 1) to determine whether a chromogenic-pathogenic relation generally existed among isolates of *B. ribis* from hosts in North Carolina; 2) to characterize the cultural variations of these isolates and those obtained from other areas; 3) to determine any relationship between culture morphology and symptomatology; and 4) to determine some

of the conditions necessary for production of the various fruiting stages.

Review of literature.—The original taxonomic description of *Botryosphaeria ribis* (5) included the achromogenic form, *B. ribis* forma *achromogena* Gross. & Dugg., and other workers confirmed these descriptions (13). The taxonomic position of the imperfect forms of *B. ribis* was not fully determined in the original description (5). Stromatic (Dothiorella), nonstromatic (Macrophoma), and microconidial stages have been described. Stromatic characteristics of the Dothiorella stage were given as similar to those of the perfect fruiting stage, whereas the Macrophoma stage reportedly may be a developmental form of the stromatic stage (13). The function of the microconidial stage apparently is not known, and successful germination of these spores is apparently not reported in the literature.

Since *B. ribis* was shown to be a pathogen of currant (16) it has been reported on a large number of woody, mostly deciduous plants throughout the world. Fenner (3) found it capable of inciting a rot of apple, and noted (3) that the chromogenic form was indistinguishable from an organism causing apple-canker in New South Wales. Those reports strengthened the contention (13) that a rot and canker of apple in South Africa, ascribed in 1919 (10) to a chromogenic species of Botryosphaeria, was caused by *B. ribis*. Extensive studies were reported in 1925 (14) and 1934 (15) on the distribution of *B. ribis* in the U. S. In some instances this fungus may have been existing as a saprophyte and not as an incitant of disease. Several reports involved pathogenicity of *B. ribis* on plants of economic importance other than apple (7, 21). Many have dealt with control and epiphytology of the apple canker and rot disease incited by *B. ribis* (6, 8, 11, 19).

Variability in culture has been ascribed to isolates of *B. ribis*, but for the achromogenic form, no distinct morphological types have been reported. Cultures of the organism are known to be difficult to distinguish from those of *Physalospora obtusa*, the incitant of black rot of apple. Both fungi commonly produce a grayish, mostly appressed, mycelial mat, becoming olivaceous or black with age. The two fungi, not infrequently found in close association in nature, have been distinguished by their distinctly different asexual spores. Asexual sporulation, however, has not always been readily obtained (5, 9, 21). Sexual sporulation of either in pure culture has been virtually unknown (5, 20).

A distinct cultural characteristic of *B. ribis* as originally described (5) was a purplish coloration induced by the fungus on starchy media. Other investigators found starchy media would differentiate chromogenic isolates of *B. ribis* (3, 4, 13), but chromogenesis was not invariably associated with pathogenicity. A yellow chromogenesis, reportedly produced in culture by isolates of *B. ribis* obtained from tung in Africa (20), has also been reported for isolates of Botryosphaeria from blueberry in the United States (17). Production of septa in germinating conidia, is a distinct characteristic ascribed to these tung and blueberry pathogens (17, 20).

Materials and methods.—The isolates of *B. ribis* used in this study are shown in Tables 1 and 2. The standard substrate for culture was potato-dextrose agar (PDA). Starch medium for chromogenic determinations contained 15 g commercial starch, 100 ml distilled water, and 3 ml saturated KH_2PO_4. The suspension was adjusted to pH 9.0 by addition of NaOH, and sterilized before plating. Simple decoction-type agars were adjusted to pH 6.5 and sterilized before plating. Plate cultures were usually maintained at room temperature and retransferred at about 10-day intervals.

Preliminary studies indicated that light was effective in inducing sporulation of *B. ribis* in culture. No isolate of this fungus was found to produce conidia in culture in the absence of light. Pycnidium-like bodies were occasionally produced by some isolates not exposed to light. Since light was a factor in inducing sporulation, fluorescent light was provided at an intensity of 220–235 ft-c at the surface of each culture plate. The illuminated area was provided with a dark background and the temperature maintained at 60°F. Cultures were arranged to enable evaluation of intensity effects.

Preliminary tests showed that fungus growth could be concisely estimated by colony diameters. Two to four such diameters were measured per test culture. Other cultural characteristics were arbitrarily coded and used to describe all cultures not grown on special substrates.

Results.—Variations in colony size and culture morphology were pronounced among isolates of *B. ribis* obtained from the same general area. Efforts were made to characterize these variations and to determine their source.

Culture variability among monoconidial isolates.—Two unrelated isolates of *B. ribis* from rotted apple fruits were grown under conditions favorable for sporulation. These 2 isolates were distinguishable on the basis of cultural characteristics. From a single pycnidium in a culture of each isolate 20 monoconidial isolates each were subsequently selected. These monoconidial isolates were grown 100 hours in plate cultures in the absence of light. Within either of these 2 monoconidial lines no significant differences in cultural characteristics were noted, even with repeated testing. Each monoconidial line retained the characteristics of the respective source isolate. This also applied to successive conidial generations derived from the original monoconidial isolates. Similarly, monoconidial isolates derived from either Dothiorella or Macrophoma fruiting bodies produced in the same culture exhibited a high degree of uniformity. Monoconidial isolates cultured in light produced both Macrophoma and Dothiorella pycnidia containing viable conidia. Hence, differences between conidial lines could not be ascribed to a given source of fruiting structure. These relationships held equally well for monoconidial isolates derived from conidia produced in nature.

Table 1.—Cultural characteristics of *B. ribis* isolated from hosts in North Carolina.

No. of isolates tested	Host source	No. of cultures per host	Mean growth of isolates[a] (mm)	No. of cultures of each type[b]			
				A	B	C	D
10	Malus	50	17.3–79.6	38	8	4	0
3	Rubus	15	38.0–64.2	14	1	0	0
2	Pyrus	10	47.1–68.5	7	3	0	0
10	Vaccinium	50	56.8–80.8	0	1	20	29
1	Prunus	5	62.3–	5	0	0	0
4	Cercis	20	83.8–90.0	20	0	0	0
30	Totals	150		84	13	24	29

Mean = 64.3 mm.
LSD = 8.8 mm**.

[a] Five colonies of each isolate were cultured 110 hours at 65° in the absence of light.
[b] Each of the 150 cultures was classified as follows: A, non-pigmented colony; B, pigmented colony, olivaceous to brown; C, pigmented colony, distinctive white margin; D, pigmented colony, yellow to reddish brown.

Culture variability among mass isolates.—The relation of cultural variability of mass isolates of *B. ribis* to the host or to geographical source of these isolates was studied in 2 tests, each repeated 4 times. In the first test (Table 1) 30 isolates were collected from 30 host plants at various locations in North Carolina. These hosts represented 6 different genera of plants commonly grown in the state. Five cultures selected from each isolate source were incubated concurrently for 110 hours at 65°F in the absence of light. The Cercis cultures as a group produced the most growth. Of the 50 Vaccinium cultures 29 produced a distinct yellow coloration. All other cultures were similar in cultural characteristics.

In the second test (Table 2) 12 isolates, from widely separated geographical locations, were the source of standardized inocula. Five cultures of each isolate were incubated concurrently for 70 hours at 65°F in the absence of light. All cultures of this group except one were of the same culture type, but a broad range in rate of growth was evident. When isolates from both tests were subjected to prolonged culture in light, pigmentation became darker.

Table 2.—Cultural characteristics of *B. ribis* isolated from hosts in widely separated geographical locations.

Host	Location	Mean Growth of isolates[a] (mm)	No. of cultures of each type[b]			
			A	B	C	D
Malus	Del.	15.2	5	0	0	0
Malus	Ind.	18.4	5	0	0	0
Malus	Va.	19.0	5	0	0	0
Malus	Del.	23.9	5	0	0	0
Aleurites	La.	24.6	5	0	0	0
Malus	Mo.	28.5	5	0	0	0
Malus	Ohio	30.7	5	0	0	0
Malus	Del.	33.3	5	0	0	0
Unknown	England	35.3	5	0	0	0
Persea	Calif.	35.5	4	0	0	1
Malus	Ind.	35.8	5	0	0	0
Malus	N. C.	36.3	5	0	0	0
		Totals	59	0	0	1

Mean = 64.3 mm
LSD = 8.8 mm**

[a] Five colonies of each isolate were cultured 70 hours at 65°F in the absence of light.
[b] Each of the 60 cultures was classified as shown in Table 1.

Culture chromogensis.—Grossenbacher and Duggar (5) found that certain isolates of *B. ribis*, grown on alkaline starch medium and exposed to sunlight, produced a distinctive purplish coloration. Some isolates used in the present study were chromogenic. Only 2 of these isolates were from Malus, but they were also atypical in other cultural characteristics. The yellow characteristic was not associated with purple chromogenesis since some of the yellow isolates were achromogenic. The yellow characteristic, also reported for *B. ribis* in Africa (20), was found by Taylor (17) to be associated with isolates of the blueberry stemblight fungus.

Sporulation studies.—*B. ribis* was grown on a number of different substrates including partially purified extracts of both host and fungus. Isolates of sexual and asexual origin were paired in culture on the above substrates, under various light and humidity conditions. None of these methods resulted in production of the perithecial stage in culture. Asexual sporulation was readily induced in culture when light was sufficient. Both Dothiorella and Macrophoma types were produced under these conditions, but neither was produced exclusively. Occasionally microconidia were produced in culture in Dothiorella-type fruiting structures. All attempts failed to germinate microconidia or to ascertain their function.

An attempt was made to correlate host or geographical source of isolates with pycnidial production or spore size. Isolates were cultured at 65°F under continuous light at 240–335 ft-c to induce sporulation. No significant correlation was found to exist between source of isolates and number of pycnidia per culture, or between number of pycnidia and the spore size. Fruiting bodies in these cultures were predominantly of the stromatic type and as large as 3–5 mm in diameter and 1–2 mm thick. Conidia produced in these cultures differed significantly in length (15.6–24.7 μ; LSD 5.3**), but not in width (5.9–7.2 μ; LSD 4.8**). Vaccinium and Cercis isolates were of the small-spored type, and 3 of the former were of the yellow-type in cultural characteristics.

Pathogenicity tests on apple fruit.—The capability of all isolates studied to induce a rot of apple tissue, and the effect of host passage on cultural characteristics, were investigated. Two thin sections each from freshly cut Golden Delicious fruits were inoculated with mycelia of each of the isolates shown in Tables 1 and 2. These sections plus control cultures of the isolates were incubated 12 days at 65°F in the absence of light. Following incubation two reisolations each were made from the above tissues and cultures. These reisolations were then incubated in both the presence and absence of light for periods commensurate with those previously used in studying cultural characteristics. All isolates induced a rot of the apple sections, and no change in cultural characteristics was noted. Three isolates that had lost the capacity to sporulate in culture were not changed in this respect by host passage.

Preparatory procedures used in the above tests might have predisposed apple tissue to rot (18). Hence, tests were repeated with entire Winesap fruits of equal maturity. Four fruits each were needle-inoculated with mycelia of each of the foregoing isolates. Inoculated fruit were incubated at 85, 75, 65, and 55°F in the absence of light, and data were taken on rate and type of rot. All isolates except one induced rot at one or more of the 4 temperatures. Most isolates induced more rot at 75 or 65°F than at 85 or 55°F. At the more favorable temperatures most isolates induced rot that was clear or colorless in external symptoms, clear or pinkish tan internally, and distinctly soft in texture. At the unfavorable temperatures these isolates induced a firm, brown rot. Some isolates failed to induce typical symptoms of bot rot, regardless of the incubation temperature. Significant grouping by host, source, or cultural characteristics in relation to rot development was not indicated. The range in external rot symptoms is illustrated in Fig. 1.

Discussion.—Monoconidial isolates of *B. ribis* derived from the same pycnidium, from different pycnidia in the same stroma, or in otherwise close proximity varied little in cultural characteristics. Mass or monoconidial isolates from different hosts or geographical locations or otherwise unrelated, frequently exhibited cultural differences, but these could not be invariably associated with a given host or geographical location. The yellow-type isolates from Vaccinium and from several hosts in other areas, however, were distinctly different from the type commonly obtained from apple in N. C. In addition, the vaccinium isolates were characteristically small-spored and did not agree with the original description of *B. ribis* (5) in that respect.

Since the Macrophoma and Dothiorella fruiting structures could be produced in the same culture by monoconidial isolates of either source, these structures were not considered independently useful as distinct taxonomic characters. Microconidia, though produced in pycnidia not unlike the macrospored types, were not produced consistently, nor was their function in the life history of the fungus established.

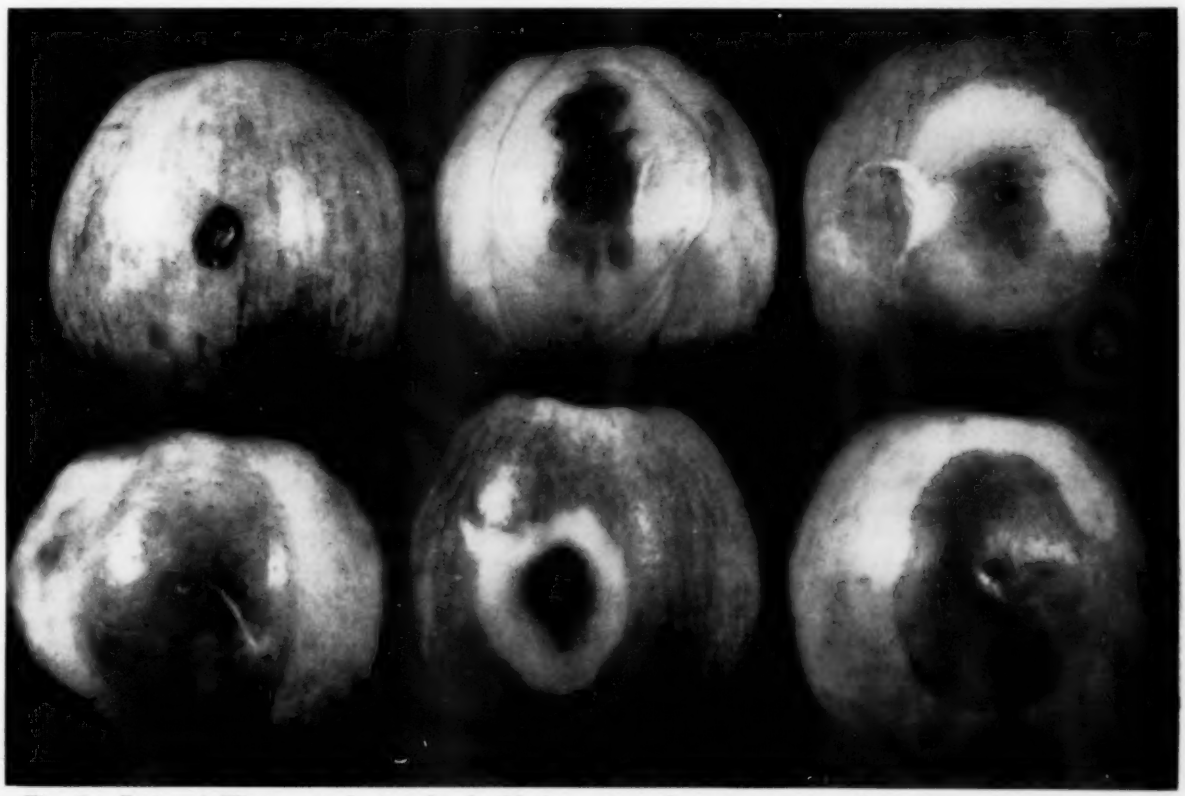

Fig. 1.—Fruits of Winesap apple inoculated with isolates of *B. ribis* and incubated 5 days at 85°F. These illustrate the range in external symptoms obtained at all temperatures under which rot development was tested.

No invariable relationship between chromogenesis on starchy media and cultural characteristics, host, or geographical origin of isolates existed among those used in this study. Most of the Vaccinium and Cercis isolates were chromogenic, but only two apple isolates were chromogenic and both of these were atypical in other respects. All isolates of *B. ribis* tested except one were capable of rotting intact apple fruits. The most characteristic rot (2) was clear in external symptoms, clear to pinkish-tan internally, and of soft texture. This type of rot usually enveloped the entire apple within 10 days at 75°F. Temperatures of 85 or 55°F were less favorable for the development of typical bot rot symptoms.—Department of Plant Pathology, North Carolina State College, Raleigh.

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SCOPOLIN PRODUCTION IN POTATO TUBERS INFECTED WITH PHYTOPHTHORA INFESTANS

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SUMMARY

Blue fluorescent zones surrounding areas of potato tissue inoculated with *Phytophthora infestans* were shown to be due to a 10–20-fold increase in scopolin. Chlorogenic acid also increased in this region, but only 2–3-fold.

Introduction.—In 1936 (3) a blue fluorescent substance later identified as scopoletin (4) (6-methoxy-7-hydroxycoumarin) was found to be associated with lesions in tobacco plants infected with tomato spotted wilt. Later, Andreae (1) showed that this compound was also responsible for the fluorescence found in potato leaves and tubers infected with leaf roll virus. More recently Uritani and Muramatsu (14) showed that scopoletin together with other coumarins and phenolic substances accumulated in sweet potatoes (*Ipomoea batatas* Lam.) infected with *Ceratostomella fimbriata*.

In these laboratories it has been frequently observed on cut tubers of potatoes (*Solanum tuberosum* L.) infected by black leg (*Erwinia atroseptica* (van Hall) Jennison) or late blight (*Phytophthora infestans* d By.), that there were, surrounding the visibly infected parts, areas that had a blue or sometimes yellowish-green fluorescence. It was of interest to see whether this fluorescence was due to local accumulations of scopoletin or other phenolic compound.

Methods.—*Inoculation and extraction of potato tissues.*—Majestic tubers were inoculated with *Phytophthora infestans* (race 4), incubated 14 days at 18°C, and cut open. A blue fluorescent zone was observed in the apparently healthy tissue surrounding the diseased region. The fluorescent zones, together with nonfluorescent controls of equal size taken from the same type of tissue on the opposite side of the longitudinal axis, were removed and weighed under methanol, and then

Table 1.—Amounts[a] of scopolin and chlorogenic acid found in potato tuber tissue, healthy and infected with *Phytophthora infestans*.

	Potato 1			Potato 2		
	Healthy	Infected	Ratio, infected: healthy	Healthy	Infected	Ratio, infected: healthy
Scopolin unhydrolyzed	0.298	1.34		.240	1.01	
Hydrolyzed	0.245	4.76	19.4	.260	3.48	13.3
Chlorogenic acid	51.5	158	3.05	74.0	149	2.02
Ratio, increase of scopoletin to chlorogenic acid		$\frac{19.4}{3.05} = 6.36$			$\frac{13.3}{2.02} = 6.58$	

[a] mg per 100 g dry matter.

exhaustively extracted with further portions of the solvent (7). The extracts were concentrated under reduced pressure and made up to standard volume.

Separation and identification of components of the extracts.—Portions of the extracts of the healthy and diseased zones were examined by two-dimensional chromatography with butanol–acetic-acid–water (6:1:2) and 6% acetic acid (5) as solvents. The two major spots from the chromatograms of both extracts were cut out, eluted with cold 70% aqueous methanol, and examined chromatographically in several solvent systems, both before and after hydrolysis, using suitable known compounds as markers (13). The ultraviolet absorption spectra of both spots, before and after hydrolysis, were determined in ethanol and 0.002*M* sodium ethoxide solution (13).

Estimation of chlorogenic acid and scopolin in the extracts.—Chlorogenic acid was estimated by measurement of the difference in optical density at 380 mμ of neutral and basic solutions of each extract (8). Scopolin was estimated, before and after hydrolysis, by measuring the fluorescence of each extract after suitable dilution at pH 5.8 (tris-maleate buffer) using "Eel" filter 621. Scopoletin fluoresces 30,000 times as strongly, weight for weight, as chlorogenic acid (Table 2), so that fluorescence from the latter substance would be negligible at the concentration found in the diluted extracts. The pH was so chosen that aesculin and aesculetin, which may be present in the extracts, fluoresced much less than scopolin and scopoletin (6).

Results.—It was concluded that one of the major spots was chlorogenic acid and the other scopolin (scopoletin-7-glucoside). These substances before hydrolysis had the same R_f values on paper chromatograms as chlorogenic acid and scopolin, and after hydrolysis the R_f values were the same as caffeic acid and scopoletin respectively. The solvent systems used were 6% acetic–acid-water, butanol-acetic–acid-water (6:1:2), butanol-water, and toluene-acetic–acid-water (4:1:5). The spectra of the eluates of these two spots before and after hydrolysis in ethanol and sodium ethoxide solution were the same as the appropriate reference compounds. Although the aglycone scopoletin has been shown to be present in the potato (1) its glucoside scopolin appears not to have been reported previously. The quantitative estimate of chlorogenic acid and scopolin in the tissue is in Table 1. Variation in the fluorescence of certain fluorescent compounds found in the potato (2) is shown in Table 2.

Discussion.—The large increase in the concentration of scopolin is obviously responsible for the blue fluorescence associated with the diseased tissue. The twofold increase observed in chlorogenic acid concentration contributed very little, because of the very much lower fluorescence of this compound. The increase in fluorescence observed on hydrolysis of the diseased extract is undoubtedly due to the formation of scopoletin from scopolin (Table 2). Goodwin and Kavanagh (6) showed a similar difference in the fluorescence of umbelliferone (7-hydroxycoumarin) and its glucoside skimmin.

The results are interesting from the plant-pathological aspect, since it has been suggested that phenolics are produced by plants in response to infection (12). Kuc (12), for example, showed that both chlorogenic and caffeic acid increased in potato tissue in response to inoculation with *Helminthosporium carbonum*, and Uritani and Muramatsu (14) found that sweet potato produced more chlorogenic acid, scopoletin, and other coumarins after inoculation with *Ceratostomella fimbriata*.

On the other hand, Johnson and Schall (10) showed that, though chlorogenic acid increased in tissue infected with virus, causing "net necrosis," a similar increase was observed in uninfected cut slices, but the amounts of unidentified phenols differed in the two cases.

All those workers found, however, that the concentration of chlorogenic acid present in diseased tissues was not sufficient to account for the plants' resistance. Kuc et al. (12) suggested that there might be a synergistic effect between chlorogenic acid and the amino acids (cf. Kirkham (11)). Uritani (14), on the other hand, found that scopoletin and certain other unidentified compounds from sweet potato had a much greater fungistatic effect than chlorogenic acid, and suggested that they might play a role in resistance.

The increases in the concentration of major phenolic compounds observed in infected plants have made it tempting to suggest that these compounds are involved in resistance. From the results presented here it is obvious that other phenolic compounds may in-

Table 2.—Fluorescence of scopoletin and other phenolic substances.

Phenolic substance	Amount needed to give the same fluorescence as 0.2μg quinine sulphate/ml (0.1*N* H_2SO_4)
Scopoletin	0.058 μg/ml
Aesculin	0.142 μg/ml
Aesculetin	1.208 μg/ml
Caffeic acid	140.0 μg/ml
Chlorogenic acid	600.0 μg/ml

crease in concentration many more times than the major substances though gross analysis would not reveal such changes. It is not suggested that such compounds are involved in resistance, but further examination of the factors initiating such changes would obviously be worth while.—National Institute of Agricultural Botany, Huntingdon Road, Cambridge, and Low Temperature Research Station, Downing Street, Cambridge.

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PHYTOPATHOLOGICAL NOTE

SURVIVAL OF STEM RUST UREDIOSPORES ON DRY FOLIAGE OF WHEAT

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Paper No. 4240, Scientific Journal Series, Minnesota Agricultural Experiment Station.

A portion of a thesis submitted in partial fulfillment of the requirements of the Ph.D. degree University of Minnesota. The writer thanks Drs. Helen Hart and E. C. Stakman for encouragement and suggestions, including suggestion of the problem. He is grateful for the use of facilities offered by the Minnesota Institute of Agriculture and the Cooperative Rust Laboratory, and for a three-year fellowship from the Egyptian Agricultural Organization, Cairo, Egypt.

Stem rust (*Puccinia graminis* Pers. var. *tritici* (Eriks. & E. Henn.) Shear, Ball, Jacks. & Stakman) urediospores carried by wind from Northern Mexico and the Southern U. S. furnish much of the inoculum for wheat grown in the Upper Mississippi Valley (3, 4). The similarity of the stem rust races throughout the Mediterranean region suggests an analogous exchange of rust urediospores between different parts of this region (2). High temperatures in the summer in the southern ranges of both these regions may not permit the urediospores to oversummer. In nearly every growing season many of the urediospores drifting or blown about by air currents eventually settle on grain fields and cereal foliage. It is not known exactly how long these spores can survive on the dry foliage before moisture is sufficient for germination and infection of the wheat, though Hwang (1) estimated that it was at least 4 days and possibly longer for spores deposited on the lower foliage and protected from direct sunlight. Swaebly (5) found that urediospores of race 15B survived 6 days on seedlings of Little Club and only 2 days on seedlings of Kentana 51A. The present study investigated survival of spores of a culture of the common subrace 139B on dry wheat leaves.

Materials and methods.—Several pots were planted with the seed of Marquis and Little Club wheats. When seedlings were 7 days old they were dusted with a mixture of talc and freshly collected urediospores of race 139B. The foliage was dry when the inoculum was applied, and the plants were subirrigated so the leaves would remain dry. Immediately, and then daily for 18 days, one pot of each variety was placed in a moist chamber and sprayed with water. Twelve hours later each pot was returned to the greenhouse. The number of pustules that developed 14 days after plants were placed in the saturated atmosphere was the criterion of survival of urediospores on the dry foliage. Temperatures averaged 87–90°F in the greenhouse in June and July, when these experiments were run; light intensity varied from 3000 to 4000 ft-c.

Results and discussion.—The number of infection centers decreased gradually with each day's delay in providing moisture for spore germination (Fig. 1). Except for a drop in the number of infection centers that happened after the 4th day, the drop happened suddenly after the 6th day for Little Club and the 7th for Marquis. In general, traces of infection occurred

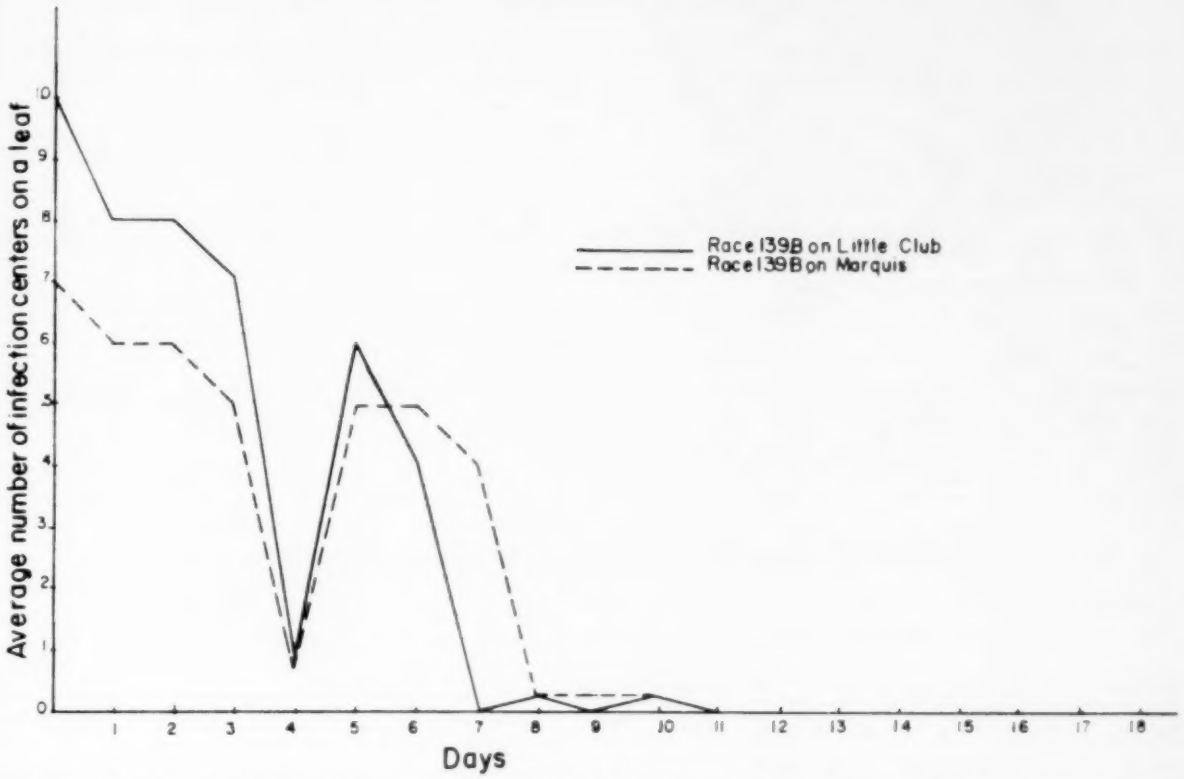

Fig. 1.—Survival of viable urediospores of race 139B on dry foliage of Marquis and Little Club wheat seedlings as judged by the number of uredia produced when moisture for spore germination was supplied after different periods.

on seedlings on which the inoculum had been exposed to summer temperatures and light for more than a week in the greenhouse. After exposure for 11 days all spores seemed to have lost viability.

It seems that the urediospores had a better chance on Marquis than on Little Club. The difference, however, was not great. The number of infection centers that appeared on Marquis was less than that on Little Club. With these two exceptions, viability of stem rust urediospores on both wheat varieties followed the same general trend.—Department of plant Pathology and Botany, Institute of Agriculture, University of Minnesota, St. Paul 1.

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BOOK LISTINGS

SCHÜETTE, K. H. ed. 1959. **Trace element problems in nature.** Issued by the Botany Department. University of Cape Town, Union of South Africa. 55 p.

This pamphlet contains 4 papers given at a symposium at the University of Cape Town. Titles and authors are: "Trace element problems in nature," K. H. Schüette; "Trace elements in the soil," Stanley S. Amdurer; "Trace elements in plants," W. A. Roach; and "Trace elements in man and animals," J. F. Brock.

Smith, J. Drew. 1959. **Fungal diseases of turf grasses.** The Sports Turf Research Institute. Bingley-Yorkshire. 90 p. 15 illustrations. Price 10/6 postpaid.

A handbook of fungal diseases of turf grasses. The author has collected relevant information on turf diseases frequently encountered in Britain. The handbook is in 2 sections: 1) The principles underlying turf disease control, including the effect of environment on disease incidence, resistant varieties, cultural practices, and control with fungicides. 2) causal organisms *per se*, symptoms, epidemiology, and detailed methods of control. The author has defined the main principles that govern the control of turf diseases and has collected information on the more important fungal diseases encountered in the British Isles. The subject matter is adequately indexed and contains a selected list of references.

Horsfall, J. G., and A. E. Dimond, ed. 1959. Plant Pathology: An advanced treatise. Vol. 1. The Diseased Plant. Academic Press, New York. 674 p. Price $22.00.

This treatise, the first of three, is the joint effort of 19 plant pathologists from 6 countries. It is addressed to the advanced student and to the established scientist. Its purpose is to examine the physiological and histological effects of disease on the plant, with few specific references to the pathogen involved. Chapter 1 discusses the concepts, terminology, and systems of disease classification; Chapter 2, the scope and contribution of plant pathology. The history of plant pathology is reviewed in chapter 3. The degree of sickness in plants and its measurement are considered in chapter 4. Chapters 5, 6, 7, 8, 9, and 10 respectively discuss the effects of disease on the integrity of plant tissue, on growth of the plant, on its reproduction, nutritional processes, water balance, and respiration. Histological defenses of the plant to disease are described in chapter 11, and physiological and biochemical defenses are described in chapter 12. Hypersensitivity and predisposition to disease are discussed in chapters 13 and 14, respectively. Physical and chemical therapy, the latter a relatively new but rapidly developing phase of plant disease control, are covered in chapter 15.

Sprague, H. B., ed. 1959. **Grasslands.** Am. Assoc. Advance. Sci. Washington 5, D. C. Publ. 53. 406 p. Price $9.00 (to AAAS members $8.00).

This interesting and valuable book surveys the role of grasslands in our national economy and gives an excellent review of our knowledge of the many aspects of grassland agriculture. It consists of 37 invitation papers, prepared by 44 specialists, presented as a part of a symposium program at the New York meeting of the American Association for the Advancement of Science, December 27–30, 1956. The areas of subject matter discussed are: 1) sciences in support of grassland research, 2) forage production in temperate humid regions, 3) engineering aspects of grassland agriculture, 4) forage utilization and related animal nutrition problems, 5) evaluation of the nutritive significance of forages, 6) grassland climatology, 7) ecology of grasslands, and 8) range management. This volume should prove helpful to the large group of scientists who desire to keep abreast of progress on the broad front of grasslands research, beyond the field of their own specialization. Chapters are included on disease and insect control, as well as a chapter emphasizing the desirability of integration of programs in genetics, pathology, and entomology.

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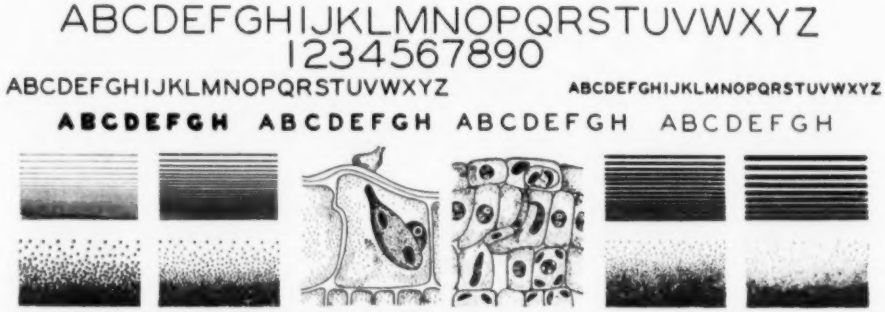

DRAWING GUIDE FOR ZINC ETCHINGS

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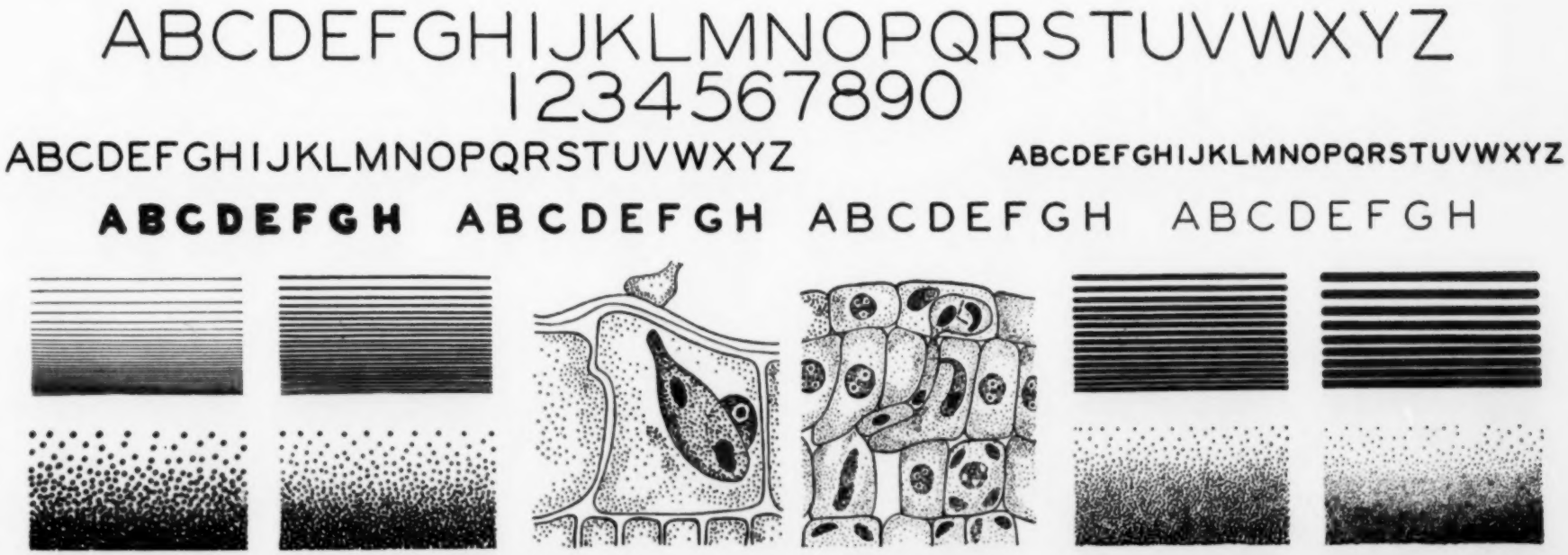

NEWS

Editor: K. W. KREITLOW, Plant Industry Station, Beltsville, Md.

The 1960 Annual Meetings of the American Phytopathological Society, the Potato Association of America, and the North Central Division of APS are being held this year at the American Baptist Assembly at Green Lake, Wisconsin, August 28-31. Facilities at the Assembly grounds are quite unique, and recreational opportunities are extensive and diversified. Wives and children are welcome, and special programs, tours, and other activities are being planned for their enjoyment. Tours of outlying research plots and other facilities are being scheduled both before and following the regular sessions. A potato tour is planned for Saturday the 27th preceding the meetings, covering the Central Wisconsin potato growing area, and including a visit to the Hancock Branch Experiment Station. Tours following the meetings will be arranged according to the wishes of those interested. These may include Fruit Disease Research at Sturgeon Bay in Door county, Forest Disease Research in the Wisconsin Rapids area, and the new Wisconsin Experiment Station Farm at Arlington, near Madison. Reservations for lodging and meals should be made directly to the Baptist Assembly, Green Lake, Wisconsin. Persons who have already requested reservations through the Madison Local Arrangements Committee also should fill out the reservation cards sent to them by their national secretaries, and mail them along with the required deposit, to the Baptist Assembly, Green Lake, Wisconsin.

Phytopathologist Raymond E. Webb has assumed his new duties as Head of the Tomato, Melon and Other Fruit Vegetable Section, Crops Research Division, Beltsville, Md. Dr. Webb replaces Dr. S. P. Doolittle who recently retired.

Plant pathologist H. Arthur Lamey, formerly stationed at Camagüey, Cuba, has been transferred to the Department of Plant Pathology, Louisiana State University, Baton Rouge where he will investigate the hoja blanca disease of rice.

The Department of Plant Pathology and Plant Breeding at the University of Georgia has recently moved into a new building, which is a part of a $12\frac{1}{2}$ million dollar science center, located on the campus in Athens, Georgia.

Phytopathologist Carl J. Eide, Department of plant Pathology and Botany, University of Minnesota was on leave of absence January 1 to April 30 to serve with the Food and Agriculture Organization of the United Nations in Santiago, Chile. Dr. Eide assisted the Chilean government in organizing a laboratory for virus disease investigations, in training technical personnel, in plant disease surveys, and in ascertaining the principal pathological problems of vegetable crops.

The International Organization of Citrus Virologists will hold their Second Conference November 7-11 at the University of Florida Citrus Experiment Station, Lake Alfred, and the U.S.D.A. Horticultural Station, Orlando. Individuals from many of the world's citrus areas are expected to attend the meetings and present research papers. The tentative program includes field trips to major growing areas and to experimental plantings.

Phytopathologist J. M. Wallace, University of California Citrus Experiment Station, Riverside, California, returned from a sabbatical leave in South America, South Africa, and Europe in mid-September. Through the assistance of a grant from the Rockefeller Foundation for travel in South America, visits were made to the citrus growing areas and research institutions of Peru, Chile, Argentina, and Brazil. Five weeks were spent in the Union of South Africa under the sponsorship of the South African Cooperative Citrus Exchange. Shorter visits were made by Dr. Wallace to the Sudan and Egypt at the invitations of the respective Ministries of Agriculture. The last two months of the leave were devoted to travel in Europe with periods of study and observation at plant virus research laboratories in Switzerland, Germany, Holland, and England.

Black spot of citrus and other citrus diseases in the Union of South Africa were studied by E. C. Calavan, Plant Pathologist of the University of California Citrus Experiment Station, Riverside, California, from February to August, 1959. Dr. Calavan worked with personnel of the various organizations conducting citrus disease research in South Africa.

Phytopathologist W. H. Sill, Jr., Kansas State University will present an invitation paper on "Soilborne virus disease of small grains in the U. S." at the International Soilborne Plant Disease Symposium to be held at the Scottish Horticultural Research Institute. Dr. Sill will also spend some time at the virus laboratories of the Rothamsted Experiment Station, England, at the invitation of Dr. F. C. Bawden, Director.

Soil microbiologist James D. Menzies, Irrigation Experiment Station, Prosser, Washington, is on leave of absence as a consultant to the Rockefeller Foundation in Chile. Dr. Menzies is investigating problems on establishing alfalfa stands.

Phytopathologist K. W. Kreitlow, Crops Research Division, Beltsville, has accepted an invitation to be Chairman of the Herbage Diseases Section at the 8th International Grassland Congress to be held in Reading, England, July 11-21. In conjunction with the Congress, Dr. Kreitlow plans to visit a number of European institutions where research is being done on forage crop diseases.

Turf grass disease control is being investigated by Paul C. Duffield at the Kansas State University. Dr. Duffield received a grant of $500.00 from The Upjohn Company for studies with fungicides.

Phytopathologist M. B. Moore, Department of Plant Pathology and Botany, University of Minnesota was named Honorary Premier Seed Grower by the Northwest Crop Improvement Association.

Blight of sugar maple is being investigated by Darroll D. Skilling at the Lake States Forest Experiment Station, St. Paul, Minn. Mr. Skilling was formerly located at the Forest Experiment Station, Wausau, Wisconsin.

Phytopathologist Leon K. Edmunds has accepted a U.S.D.A. position to investigate sorghum diseases at Kansas State University, Manhattan.

Plant pathologist W. C. Normand has accepted a research position in the Louisiana State University Department of Botany, Bacteriology and Plant Pathology to work on the physiology of sugarcane.

Lake S. Gill, Chief, Division of Forest Disease Research at the Rocky Mountain Forest and Range Experiment Station in Fort Collins, Colorado, retired February 29, after more than 35 years with the U.S.D.A. Dr. Gill is an authority on dwarf mistletoe; his latest work is a review of the literature on mistletoe.

Deaths—Floyd D. Bailey died March 12 in Corvallis, Oregon, at the age of 72. Mr. Bailey joined the Oregon Agricultural Experiment Station in 1911. He later served as a plant pathologist in the U.S.D.A. Food and Drug Administration for more than 25 years until retirement in 1950.

Louis O. Kunkel died March 20 at the age of 75. Dr. Kunkel was a Member Emeritus of the Rockefeller Institute having retired in 1949. He was best known for his pioneering research on virus diseases particularly the aster yellows virus.